Ascochyta blights of grain legumes

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Cover photos:
From top to bottom: Ascochyta blight (*Didymella rabiei*) on chickpea leaflets; Faba bean seeds infected by *Ascochyta fabae*; *Mycosphaerella pinodes* colony on Petri dish; Ascochyta blight (*Mycosphaerella pinodes*) on pea; Pycnidia of *Ascochyta fabae* in leaf tissue

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Foreword

Robert (Bob) A. Henson

We dedicate this special issue to our friend and colleague Dr. Bob Henson who unexpectedly passed away during the first international workshop on Ascochyta blight entitled 'Ascochyta 2006.' Dr. Henson of Carrington, North Dakota, USA was noted for his research on management for Ascochyta blight and was also instrumental in establishment of mist nurseries for evaluation of several crops for resistance to Sclerotinia white mold in collaboration with plant breeders. Dr. Henson's degrees include a B.A. in Chemistry from Macalester College in St. Paul, Minnesota, and a Master of Agriculture in Plant and Soil Technology and Ph.D. in Agronomy from the University of Minnesota, St. Paul. He was an active member of the American Society of Agronomy, the Crop Science Society of America and the Soil Science Society of America as well as numerous industry organizations. He was a member of the North American Pulse Improvement Association and was currently serving on the Board of Directors. Prior to joining the Carrington Research and Extension Center in 1998, he worked as a bean Agronomist and Physiologist in Brazil and Ecuador and as a consultant to the World Bank in Mexico and Bolivia. Bob was well recognized as a hard worker and productive researcher with a friendly outgoing manner and smile for everyone. He is survived by his wife Soraia, two sons, Robert and Peter, and a daughter, Gabriella. Bob was a good friend of the Ascochyta and Sclerotinia communities and will be sorely missed.

Robert (Bob) A. Henson

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Ascochyta blights of grain legumes

Ascochyta blights of the cool season food legumes (peas, lentils, chickpeas and faba beans) are important production constraints in all regions where these crops are grown and in severe cases result in complete crop loss. The global importance of Ascochyta as a production constraint to these legumes is well documented. To review past research and to develop plans for overcoming this production constraint, the first international workshop on Ascochyta blight entitled 'Ascochyta 2006' was organized and conducted at Le Tronchet, France during the week of 2–6 July 2006. The workshop brought together experts on all aspects of the problem to review current knowledge and to formulate plans for future research and collaboration. Plenary sessions were devoted to pathogen biology, plant resistance, epidemiology and integrated disease management. Presentations of posters of current research complimented these sessions and provided additional insights into the disease. Four additional talks followed by informal round tables were given in order to enlarge the thematic sessions: interest in *Medicago truncatula* for disease resistance in grain legumes(Alain Baranger, INRA, France); the *Ascochyta* genus (Ivan Sache, INRA, France); grain legume research and extension (Robert Morrall, University of Saskatchewan, Canada); the place of legumes in crop rotations (Raphaël Charles, University of Changins, Switzerland). The goal of the workshop was to identify gaps in knowledge, identify new research approaches and to establish collaborative relationships among Ascochyta blight researchers. Sixty-five participants from 13 countries were in attendance. Most of the participants reported on their current research on Ascochyta on one or more of the cool season food legumes.

The workshop was very successful in achieving its goals set out by its organizers, and the participants enjoyed the venue and hospitality provided by the local organizing committee. This special issue will serve as a useful reference for years to come. Recommendations of the group were formulated to stimulate future collaborative research on the Ascochyta blight problem as it affects the cool season food legumes. A committee was established for developing a follow-up workshop to be held at Washington State University in Pullman, Washington, USA in June of 2009 entitled 'Ascochyta 2009.'

This special issue of EJPP contains invited presentations and contributed papers by workshop participants. The workshop was organized by INRA (French National Institute of Agronomical Research), Agrocampus Rennes (College of Agronomy), AEP (European Association of Grain Legumes), USDA (United States Department of Agriculture) and SFP (French Plant Pathology Society).

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Towards identifying pathogenic determinants of the chickpea pathogen *Ascochyta rabiei*

David White · Weidong Chen

Abstract Ascochyta blight is a serious disease of cool-season grain legumes (chickpea, faba bean, lentil and pea) caused by fungal species of the anamorphic genus Ascochyta and related genera. Despite extensive studies on the biology, ecology, epidemiology and management of the disease, little is known about the pathogenic determinants of these pathogens. This research aims at using *Ascochyta rabiei* as a model for the genus in investigating genetic factors of pathogenicity, with the ultimate goal of elucidating pathogenic mechanisms. Three advances were made: (1) insertional mutants with altered pathogenicity were identified through in vivo screening, and genomic regions adjacent to the insertion sites in selected mutants were determined; (2) a phage library of *A. rabiei* genomic DNA was constructed, and the library was estimated to provide complete coverage of the *A. rabiei* genome. This library was used successfully to recover clones with DNA adjacent to insertional mutation sites and to isolate specific genes; (3) DNA probes specific for an acyl-CoA ligase (*cps*1) and a polyketide synthase gene (*pks*1) were developed and library clones containing the corresponding genomic regions were identified from the phage library. These advances provide the foundation and necessary tools for experimentation of ectopic complementation assays and targeted mutagenesis to elucidate the genetic mechanisms of pathogenicity of *A. rabiei*.

Keywords *Agrobacterium*-mediated transformation · Gene disruption · Phage library

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Introduction

Ascochyta blight is an important disease of cool-season grain legume crops including chickpea, faba bean, lentil, and pea. The pathogens are often host-specific, each species causing the disease with economical significance only on specific crops, e.g. *Ascochyta rabiei* on chickpea, *A. fabae* on faba bean, *A. lentis* on lentil, and *A. pisi* *Mycosphaerella pinodes*, and *Phoma medicaginis* var. *pinodella* on pea (Peever 2007). Extensive studies have been conducted on a number of the species on pathogen ecology (Taylor and Ford 2007), epidemiology and management (Tivoli and

Banniza 2007; Davidson and Kimber 2007). However, research on pathogenic determinants of *Ascochyta* spp. in general has received little attention.

Ascochyta blight pathogens are all necrotrophic, killing plant cells in advance of mycelial development. Therefore, toxins and cell-wall degrading enzymes are often presumed to be important biochemical determinants of pathogenesis. Among the ascochyta pathogens, *A. rabiei* on chickpea is probably the most intensively studied pathosystem in terms of biochemical interactions between the host and the pathogen. *Ascochyta rabiei*, causal agent of chickpea ascochyta blight, produces toxin solanopyrones through the polyketide synthesis pathway (Alam et al. 1989; Hohl et al. 1991), and hydrolytic or cell-wall degrading enzymes (Tenhaken and Barz 1991; Tenhaken et al. 1997). Several lines of evidence show the roles of the phytotoxins in causing blight (Chen and Strange 1991; Kaur 1995). The hydrolytic enzymes are considered necessary for fungal nutrition and to facilitate spatial spread of fungi (Walton 1994). *Ascochyta rabiei* was first transformed with the protoplast/PEG protocol with a GUS reporter gene for observing the infection process (Kohler et al. 1995), and later transformed with *Agrobacterium*-mediated transformation (AMT) for studying pathogenicity factors (White and Chen 2006; Morgensen et al. 2006). However, little information is currently available about the genetic determinants of pathogenicity of the ascochyta pathogens. Knowledge of pathogenic determinants will allow us to develop a better understanding of host-pathogen interactions to devise novel or more effective measures in managing the disease.

There are two approaches to investigate pathogenic determinants of fungal pathogens. One is targeted gene disruption to construct mutants defective of a defined gene of interest. In this instance, target genes could be previously-reported pathogenicity genes in other pathosystems. The role of the gene in infection can be assessed by comparing the pathogenicity of the mutant with that of the wild-type. The other approach is to generate random and tagged mutations within the pathogen genome. The modern technique in this approach is through insertional mutagenesis of either restriction-enzyme-mediated integration (Oliver and Osbourn 1995; Kahmann and Basse 1999) or AMT (Michielse et al. 2005). This approach does not require a priori knowledge of gene function, and it involves generation of a library of random mutations, screening the library for altered phenotypes or pathogenicity, and characterization of disrupted genomic regions. This strategy is powerful in the identification of previously unknown pathogenicity factors.

There are a number of previously reported conserved fungal virulence factors that could be explored in *A. rabiei*. Lu et al. (2003) described a general fungal virulence factor (an acyl-CoA ligase *cps*1) in several plant-pathogenic ascomycetes. Disruption of the *cps*1 homolog in several plant pathogens produced no observable growth phenotype, but showed reduced virulence. Production of melanin has also been shown to be a virulence factor in some pathogenic fungi (Henson et al. 1999; Kawamura et al. 1999). *Ascochyta rabiei* produces melanin through the 1,8-dihydroxynaphthalene pathway via polyketide synthesis (Chen et al. 2004b). Thus, polyketide synthases could potentially be pathogenicity factors in *A. rabiei* through their involvement in melanin biosynthesis or in the synthesis of phytotoxin solanapyrones (Hohl et al. 1991).

The goal of our research is to use *A. rabiei* as a model for the other ascochyta pathogens to open the door for investigating pathogenic determinants. Our research hypotheses are (1) insertional mutagenesis can be applied to *A. rabiei* to elucidate pathogenic determinants, and (2) some of the previously-reported pathogenicity factors from other plant pathogens could be identified and isolated from *A. rabiei*. Here we report successful identification and characterization of tagged mutants with reduced or lost pathogenicity, development of gene-specific probes, construction of a phage library of the *A. rabiei* genome, and the isolation of clones containing potential pathogenicity factors through screening the library. The research provides the foundation and necessary tools for further assessing the roles of the respective genes in causing ascochyta blight.

Materials and methods

Fungal strains, transformation, and pathogenicity screening of transformants

The pathotype II strain AR628 (Chen et al. 2004a) of *A. rabiei* was used in transformation experiments. The transformation was carried out as previously described (White and Chen 2006). Briefly, conidia of strain AR628 were co-cultured with cells of *Agrobacterium tumefaciens* carrying T-DNA. The co-cultivation was spread on a membrane and incubated on medium containing timentin and hygromycin to select against bacteria and select for hygromycin-resistant transformants. Transformed conidia that grew on the selective medium were further purified by single-conidium isolation. After confirming resistance to hygromycin, the transformants were screened for altered pathogenicity before further characterization.

The mini-dome bioassay (Chen et al. 2005) was used to screen transformants for reduced pathogenicity. The transformants were always compared with wild-type strains AR19 (pathotype I) and AR628 in the pathogenicity assays on chickpea cvs Dwelley and Spanish White (six plants in three replicates of each cultivar per strain). Disease severity was assessed according to the 1–9 rating scale (Chen et al. 2004a). The transformants that showed reduced pathogenicity in the first assay were tested again in a second assay. Nine transformants that showed significantly reduced pathogenicity in both assays were selected for further study. In addition, two transformants that lost ability to produce conidia were also selected for further characterization.

Southern hybridization, inverse-PCR and sequence analysis of transformants

Southern hybridization was used to determine the number of insertions in transformants. Genomic DNA from transformants and wild-type AR628 were digested with XhoI (New England Biolabs, Ipswich, MA, USA), separated on an agarose gel, and transferred to a nylon membrane. A DIG-labelled DNA probe was synthesized from an internal region of the hygromycin B gene using PCR (White and Chen 2006). Probed membranes were processed according to the manufacturer's instructions, and detected using the anti-DIG-alkaline phosphatase conjugate antibody and the chemiluminescent substrate CSPD (Roche) by exposure to autoradiograph film to visualize hybridized fragments.

To isolate DNA regions flanking the insertion sites in the transformants, an inverse-PCR technique was used. Genomic DNA of selected transformants was digested with XhoI to isolate DNA flanking the right border, and digested with either SacI, SalI or KpnI to isolate DNA flanking the left border. Digested DNA was ligated to itself and used as template for the inverse PCR using primers LB5IP and RB5IP (White and Chen 2006). Products were isolated from agarose gels and ligated to the pGEM-T Easy vector (Promega, Madison, WI, USA) for further analysis.

To determine the gene disrupted by the T-DNA in each transformant, the ends of each cloned inverse-PCR product were sequenced using the M13F (-20) and M13R (-21) primers (New England Biolabs). Forward and reverse sequences were joined after removing all vector and T-DNA border sequences. Assembled sequences were compared to each other to verify that each contained a unique region of the *A. rabiei* genome and translated in all six reading frames for comparison to the GenBank database as well as the *Stagonospora nodorum* genome (http://www.broad.mit.edu). The *S. nodorum* genome was selected because, for the fungi with genomes available, *S. nodorum* is the closest phylogenetically related to *A. rabiei* (Peever et al. 2007).

Development of gene-specific probes for *A. rabiei*

Specific probes were developed for genes that could be potential virulence factors in *A. rabiei*. The genes encoding the polyketide synthase (*pks*1) from *Glarea lozoyensis* (Zhang et al. 2003) and an acyl-CoA ligase (*cps*1) from *Cochliobolus heterostrophus* (Lu et al. 2003) were selected as candidate virulence factors. These gene products were compared to and aligned with

the translated genome of *S. nodorum*, and conserved locations were selected to design PCR primers. PCR primers pksF2 (5′-CAC-TACCACTGCCGTCGCAT) and pksR2 (5′-TAGACTTGACCATGCCACTGCA) were designed to amplify a 562-bp region of the *pks*1 gene, and primers cpsF (5′-GGGACAAGAG-CAACCTCTA) and cpsR (5′-TGGTAGTTG-TATGCAGC) to amplify a 683-bp region of the *cps*1 gene. PCR products were cloned into the pGEM-T Easy vector (Promega) and sequenced using the M13F and M13R primers as described above.

Construction and screening of a genomic library of *A. rabiei*

To construct a genomic library of *A. rabiei*, genomic DNA of strain AR628 was digested with ApoI and fragments between 7,000 and 10,000 bp were eluted from agarose gels, desalted, and ligated to pre-digested and phosphatased (*Eco*RI) Lambda ZAPII vector arms (Stratagene, La Jolla, CA, USA), packaged using Gigapack III extracts, and amplified in *E. coli* strain XL1-Blue. The efficiency of the ligation and packaging reactions were determined using X-GAL and IPTG. To determine the average insert size of the recombinant phage library, plasmid rescue (in vivo excision) was performed on phage collected from ten random plaques using the *ExAssist*® helper phage and *E. coli* strain SOL-R. Recovered plasmids were digested with ApoI and separated on an agarose gel. A single round of library amplification was performed and the phage suspension stored in 7% DMSO at –80°C until use.

To isolate clones from the library that contain either *pks*1 or *cps*1 homologs, probes were constructed using PCR with the *pks* or *cps* primers and labelling procedures described above. Approximately 80,000 plaques were transferred from NZY agar to nylon membranes (Amersham) and probed sequentially, first with the *cps*1 probe, then with the *pks*1 probe. Single plaques that hybridized with each probe were recovered from the corresponding NZY plate and in vivo excision reactions were performed to rescue phagemid DNA. Recovered phagemid DNA was used as template for PCR with the corre-

sponding primer pairs used to generate the probe, and were also digested with ApoI to estimate the insert size by agarose gel electrophoresis.

To isolate clones from the phage library that contain DNA adjacent to the T-DNA insertion sites, approximately 80,000 plaques were screened with probes generated from inverse-PCR products. Probes from the transformants were mixed together for the primary hybridization and detection screen. Phage from positive plaques from the primary screen was harvested and pooled in SM buffer to make an enriched phage stock for infecting *E. coli* XL-1 Blue cells. Plaques generated from the enriched phage stock were transferred to nylon membranes and screened with individual probes. Phagemids were recovered by in vivo excision as described above from phage collected from three plaques identified by each probe and analyzed by restriction digestion and sequencing.

Results

Identification and characterization of transformants with altered pathogenicity

Approximately 800 transformants were generated and screened for pathogenicity in this study. The transformants exhibited a wide range of variation in colony morphology, growth rate, and conidial production. For example, six transformants had lost ability to produce conidia. Some transformants produced constitutively black mycelium. In general, under selection conditions (V8 agar with 200 μg ml^{-1} hygromycin) many transformants produced less conidia than when grown in the absence of selection (V8 agar without hygromycin). The wild-type strain AR628 consistently produced about 3.7×10^7 conidia per plate, 63 transformants produced about 10% of conidia of WT, seven transformants produced about 1.5% of conidia of the wild type. Two transformants produced 5× more conidia than the wild type.

In pathogenicity screening, the transformants that lost ability to produce conidia were not screened because the screening procedure uses conidia as inoculum (Chen et al. 2004a). Most of the transformants screened were about equally

virulent as the parental wild-type strain, producing disease scores above 6 (Fig. 1). Some transformants showed reduced pathogenicity in the first screening, but produced higher levels of disease severity in a second pathogenicity assay possibly due to heterogeneity of nuclei (co-existence of transformed and non-transformed nuclei). To date, 21 of the transformants produced significantly lower disease severity (score <4 on a 1–9 rating scale) than that of the wild-type in at least two independent pathogenicity assays (Fig. 1). Nine of the 21 transformants plus two transformants that lost ability to produce conidia were selected for further characterization (Table 1).

Southern hybridization, inverse PCR and sequence analysis of selected transformants

Southern hybridization of digested transformant DNA probed with the hygromycin-resistance gene (*hph*) showed single hybridization bands of various sizes (data not shown), confirming that the T-DNA was integrated into the genome of *A. rabiei* and that each transformant contained a single insertion. Inverse PCR amplified single products from transformants, ranging in size from 850–2500 bp (Table 1). Sequences adjacent to the insertion sites from the transformants were first compared among themselves, and comparison showed that two pairs of the 11 transformants, ArW520 vs ArW525, and ArW247 vs ArW251, were identical in insertion locations. This reduced the number of characterized transformants from 11 to 9.

The sequences flanking the T-DNA from each transformant were used as queries in tBLASTx searches of the GenBank database as well as the genome database of *S. nodorum*. DNA recovered from two (ArW8 and ArW540) of the transformants shared a high degree of similarity with known proteins while the sequence from another transformant (ArW247/ArW251) shared significant similarity with a hypothetical protein of *A. nidulans* (Table 1). The translated DNA (576 bp) from transformant ArW8 shared 71% identity (91/128 aa) with the kinesin of *C. heterostrophus* (accession AY230433). Translated DNA from transformant ArW540 (440 bp) shared 66% identity (86/130 aa) with the transposase protein of the *S. nodorum* transposon *molly*. Three additional sequences shared minimal sequence similarity with proteins in the database as indicated by the low E values (Table 1). The remaining three sequences (from transformants ArW522, ArW524 and ArW529) did not have any similarity to known proteins (Table 1). In searching the *S. nodorum* genome, sequences of three transformants (ArW8, ArW247 and ArW540) shared significant similarity to translated regions (hypothetical proteins) of the genome, while sequences of the remaining six transformants did not have any similarity with any translated region of the genome (Table 1).

Construction of genomic library and screening with gene-specific and transformant-generated probes

A phage library consisting of 1.7×10^6 recombinants containing *A. rabiei* DNA was constructed with a background (phage without insertion) of less than 2%. The average insert size of the recombinants was about 6,300 bp (data not shown). Thus, this DNA library contains more

Fig. 1 Screening transformants for altered pathogenicity using a mini-dome bioassay. 1 = non-inoculated control; 2 = inoculated with parental wild-type strain AR628; 3, 4 and 5 = transformants ArW1, ArW8 and ArW16, respectively
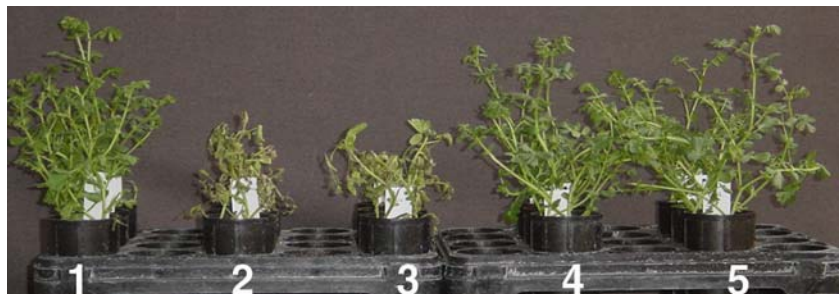
Table 1 Characterization of insertion sites in selected transformants of *A. rabiei*

| Strain	Disease score[a]	Inverse PCR[b]	Sequence length[c]	GenBank tBLAST results (Accession #)	E value	S. nodorum genome	E value
ArW8	3.5 ± 0.5	1900	576	*C. heterostrophus* kinesin (AY230433)	$1e^{-46}$	SNOG_04288: hyp. protein	$2e^{-44}$
ArW247/ArW251	NT[d]	500	175	*A. nidulans* hypothetical protein (XM_659106)	$3e^{-10}$	SNOG_01574 hyp. protein	$5e^{-16}$
ArW519	2.0 ± 0.5	1600	433	*M. musculus* p21 activated kinase (AK08851)	0.096	No significant similarity	–
ArW520/ ArW525	1.3 ± 0.3	850	786	*A. erytherum* put. transcript. rep. (AY62365)	0.051	No significant similarity	–
ArW522	1.2 ± 0.3	2200	742	No significant similarity	–	No significant similarity	–
ArW524	2.5 ± 0.9	1500	755	No significant similarity	–	No significant similarity	–
ArW529	1.0 ± 0	2100	818	No significant similarity	–	No significant similarity	–
ArW540	1.8 ± 0.6	2500	440	*S. nodorum* transposon *molly* (AJ488502)	$3e^{-34}$	SNOG_08250 hyp. protein	$8e^{-10}$
ArW541	1.2 ± 0.3	2300	619	*A. oryzae* cDNA, contig sequence: (AoEST1849)	6.6	No significant similarity	–
Ar628 (WT)	8.0 ± 0.8	–[e]	–	–	–	–	–

[a] Pathogenicity score (± standard deviation, n=3) on chickpea cv. Dwelley using a 1–9 rating scale (1 = healthy, no disease and 9 = dead plant)

[b] Size in base pairs of total product of inverse PCR

[c] Sequence length used in tBlast searches

[d] Not tested due to lack of conidia

[e] Not Applicable
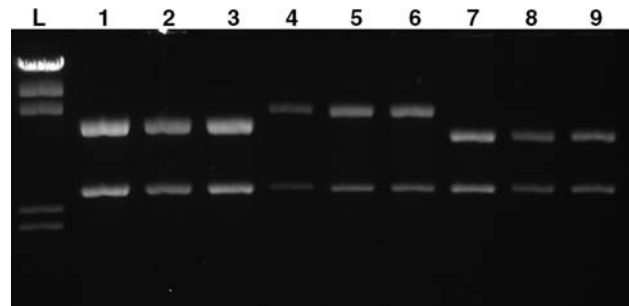
Fig. 2 DNA inserts rescued from positive plaques (three random selected plaques per probe), Lane L, λDNA digested with HinDIII; Lanes 1–3, plaques positive with the ArW8 probe; Lanes 4–6, plaques positive with the ArW519 probe; Lanes 7–9, plaques positive with the ArW541 probe

than 10,000 MB of *A. rabiei* DNA. Assuming a genome size of 40 MB for *A. rabiei* (Akamatsu and Peever 2005), this library would provide more than 250× coverage. After a single round of amplification the final titer of the library was 1×10^9 pfu ml^{-1}.

Screening with gene-specific probes

The amplified *pks*1 DNA fragment had 81% (455/562) identity to the *Bipolaris oryzae* polyketide synthase gene (accession AB176546). The amplified *cps*1 fragment was 82% (560/683) identical to the *cps*1 gene (accession AF332878) of *C. heterostrophus*. The two sequences were deposited into GenBank and assigned the accession numbers EF092313 (*ARcps*1) and EF092314 (*ARpks*1). Two positive clones were identified with the *pks* probe, and the two clones contained 5,800- and 7,000-bp inserts, respectively. The 7,000-bp clone contained an intact DNA region defined by the two PCR primers pksF2 and pksR2. A single positive clone with 5,500-bp insert was identified with the *cps* probe and it contained the entire DNA region defined by the *cps* primers cpsF and cpsR (data not shown).

Screening with transformant-generated probes

After a primary screening of approximately 80,000 plaques with a mixture of the nine probes of the transformants, phage from 55 positive plaques was harvested and pooled to form an enriched phage stock for a secondary screen using individual probes. Each probe in the secondary screening was exposed to approximately 30,000 plaques generated from the enriched phage stock. Phagemid DNAs were rescued from three random plaques identified by each single probe and in each case the three recovered phagemids contained the same sized-DNA inserts (Fig. 2). It was assumed that the three clones represented the same region of *A. rabiei* genomic DNA and only one clone was selected for further analysis.

Discussion

Three important advances were made towards identifying pathogenicity determinants of *A. rabiei*. First, insertional mutants with altered pathogenicity were identified through in vivo pathogenicity assays, and the DNA sequences adjacent to insertion sites were determined. Second, a phage DNA library of *A. rabiei* was constructed with about 250× coverage of *A. rabiei* DNA. Hybridization with either gene-specific probes or probes generated from random insertion sites of transformants always identified positive clones in the library, proving its utility in isolating other genes. Third, probes for specific genes (*cps*1 and *pks*1) with the potential of being general pathogenic determinants in *A. rabiei* were developed, and positive library clones were identified through Southern hybridization. The positive clones containing the specific genes or the insertion sites will be useful for either ectopic complementation tests or targeted mutagenesis.

Insertion in one of the transformants appears to be within a known fungal gene. Transformant ArW8 is less pathogenic (Table 1), and the T-DNA has disrupted a kinesin-like gene. Kinesins play important roles in the transport of cell organelles, polarized growth, and secretion (Schoch et al. 2003), and the kinesins of the yeast *Schizosaccharomyces pombi* as well as the corn smut fungus *Ustilago maydis* have been studied extensively (Steinberg and Fuchs 2004; Straube et al. 2006). However, this is the first report of a kinesin-like gene potentially being involved in plant pathogenesis. Its role remains to be confirmed and its mechanisms in pathogenesis are not clear.

Diverse DNA sequences are found in the insertion sites, showing the randomness of the insertion mutagenesis. Pathogenesis is a complex biological process involving diverse factors. Pathogenesis of the necrotrophic pathogen *A. rabiei* is predicted to involve a number of processes including attachment and penetration of host plant tissue, as well as production and secretion of extracellular enzymes and phytotoxins, and each process is likely to be controlled by several genes. A mutation in any gene involved in these processes could result in altered pathogenicity.

Many of the sequences recovered from the nine transformants had no significant matches either in the GenBank database or within the *S. nodorum* genome, and the significant *in silico* similarity identified in three of the transformants is primarily with hypothetical proteins. This inability to detect any known sequences with significant similarity could be due to the limited length of query sequences available, to the fact that they are unique pathogenicity factors in *A. rabiei*, or to the fact that small deletions of genomic DNA occurred during T-DNA integration events (Bundock and Hooykaas 1996), resulting in the loss of a coding region flanking the insertion.

Gene-specific probes were developed to isolate a polyketide synthase gene and an acyl-CoA ligase gene from the *A. rabiei* library. These genes were selected because they were shown to be conserved pathogenicity factors in other pathosystems (Kawamura et al. 1999; Lu et al. 2003). Screening using these gene-specific probes also served the purpose to test the completeness and usefulness of the DNA library. Both probes identified positive plaques in the library despite the fact only a portion (80,000 plaques) of the library was exposed to the probes. Thus, this library should be useful for isolating other genes of interest and it will be a valuable resource available to the scientific community for studying *A. rabiei* or other related plant pathogens.

Two pairs of transformants were shown to be identical, likely to have resulted from conidia of the same transformation events, since they were isolated from the same transformation membrane. Although the unintentional inclusion of these transformants resulted in redundancy of work, in retrospect, it provided an important internal control. This result showed that the characterization procedures from pathogenicity screening to inverse PCR are reliable and reproducible, giving us confidence in the techniques developed in this study.

Two approaches need to be taken to unequivocally demonstrate the roles of the identified potential pathogenicity determinants in *A. rabiei*. One approach is to use ectopic complementation tests to prove the role of the genes disrupted in the random insertional mutants. A second shuttle vector carrying the *nptII* gene for geneticin resistance expressed by the *A. nidulans trpC* promoter has been created for delivering library DNA via AMT to hygromycin-resistant transformants, and selection on hygromycin and geneticin has been shown to be stable (unpublished). Additionally, *A. rabiei* is heterothallic (Trapero-Casas and Kaiser 1992). Thus segregation analysis could also be employed.

Another approach is to carry out targeted mutagenesis specifically on the *cps* and *pks* genes to create knockout mutants. To create deletions in the *pks* and *cps* library fragments, a short region of each clone has been removed by restriction digest and replaced with the *trpC-hph* antibiotic resistance cassette. Disruption cassettes containing library clones in a markerless T-DNA shuttle vector are being constructed for delivery into *A. rabiei* wild-type strains via AMT. Integration can occur at the genomic site of interest (homologous recombination) or at other sites (illegitimate recombination), which would be

distinguished by PCR or Southern hybridization. Since transformation may induce unexpected changes in chromosome structure or complement, it is prudent to evaluate the phenotypes including pathogenicity of a number of independent transformants including those that have not undergone disruption at the gene of interest.

Despite significant advances in understanding genetic factors of pathogenicity in a number of phytopathogenic fungi (e.g., Gilbert et al. 2004; Talbot 2004), little information is available about pathogenicity determinants in *Ascochyta* spp. Using *A. rabiei* as a model for *Ascochyta* and other closely related plant pathogens, the research presented here showed the feasibility of and provided necessary tools for studying pathogenicity determinants in ascochyta blight pathogens of grain legumes. A detailed knowledge of pathogenic determinants of *A. rabiei* and of chickpea resistance response (Cho et al. 2005; Coram and Pang 2006) will be invaluable in developing our understanding of the interaction between *A. rabiei* and chickpea, and in devising novel or more effective measures in managing the disease. The information may also be applicable to ascochyta blight of other cool-season grain legumes.

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FULL RESEARCH PAPER

Biotic factors affecting the expression of partial resistance in pea to ascochyta blight in a detached stipule assay

Caroline Onfroy · Alain Baranger · Bernard Tivoli

Abstract The expression of partial resistance in pea to ascochyta blight (caused by *Mycosphaerella pinodes*) was studied in a detached stipule assay by quantifying two resistance components (fleck coalescence and lesion expansion) using the method of point inoculation of stipules. Factors determining optimal conditions for the observation of partial resistance are spore concentration, the age of the fungal culture prior to spore harvest and the pathogenicity of the isolate used for testing. Partial resistance was not expressed when spore concentration was high or when the selected isolate was aggressive. Furthermore, assessments of components of partial resistance were highly correlated with disease severity in a seedling test. A screening protocol was developed based on inoculations of detached stipules to study partial resistance in pea. To simplify the rating process, a more comprehensive disease rating scale which took into account fleck coalescence and lesion expansion was tested by screening a large number of genotypes.

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Introduction

Ascochyta blight of pea (*Pisum sativum*) is caused by three related fungal species, commonly referred to as the Ascochyta complex: *Ascochyta pisi*, *Ascochyta pinodes* (teleomorph: *Mycosphaerella pinodes*) and *Phoma medicaginis* var. *pinodella*, formerly known as *Ascochyta pinodella* (Jones 1927). *Mycosphaerella pinodes* and *P. medicaginis* var. *pinodella* cause foot rot, and similar symptoms on leaves, stems, pods and seeds (Hare and Walker 1944) which can result in substantial yield and seed quality losses in France (Allard et al. 1993) and throughout the major pea cropping regions worldwide (Bretag and Ramsey 2001). The first studies on pea resistance to *M. pinodes* have shown the absence of specific resistance (Nasir et al. 1992; Clulow et al. 1992). Most recent studies on resistance to the ascochyta blight complex in pea have described the observed resistance as partial (Onfroy et al. 1999; Wroth and Khan 1999; Wang et al. 2000; Xue and Warkentin 2001;

Timmerman et al. 2002; Prioul et al. 2003, 2004; Fondevilla et al. 2005). Partial disease resistance is defined as an interference with one or more steps of the epidemic cycle, resulting in a slow-down of disease progress and/or a reduction in the pathogen multiplication (Parlevliet 1979). The growth of the pathogen can be assessed quantitatively both by directly assessing disease severity (symptoms) and disease development over time, or by considering disease severity as the result of different factors (Parlevliet 1979). These epidemiological components of quantitative resistance include resistance to infection (*i.e.,* reduced germination, appressorium formation or penetration), delayed incubation period (from inoculation to the occurrence of the first symptoms), delayed latency period (from inoculation to sporulation), reduced infectious period (sporulation duration), and reduced intensity of spore production (spore quantity per time unit).

Specific and reliable methodologies are needed for the assessment of these components of resistance under field or controlled conditions. The use of point inoculation on leaflets, either detached or in situ under controlled conditions, can be helpful in dissecting plant reactions and for providing insight into the different steps of the epidemic cycle. In the *Botrytis fabae*/faba bean pathosystem, Tivoli et al. (1986) used a detached leaf assay to determine three main epidemic phases, namely appearance of symptoms (number of spots 15 h after inoculation, rate of new spot formation), disease development (disease severity score 6 days after inoculation), and sporulation (number of spores/leaflet 11 days after inoculation). More recently, Bouhassan et al. (2003), using this methodology in the same pathosystem, quantified five components of partial resistance: the incubation period, the number of spots, lesion diameter, the latency period and the intensity level of sporulation.

Few references pertaining to the use of point inoculation of leaves to study ascochyta blight on pea are available. Heath and Wood (1969) used excised leaves to determine the factors acting on the phases of the epidemic cycles of *M. pinodes* and *A. pisi* (spore concentration, leaf age, water content of the leaf). This method has also been used to screen for cultivar susceptibility and/or pathogenicity of isolates. Wang et al. (2000), using excised leaves to study suscep-tibility in pea to *A. pisi*, reported significant

isolate × genotype interactions. Based on point inoculation of leaves in situ on plants, Nasir et al. (1992) described the development of different *M. pin-odes* pathotype groups in susceptible and partially resistant pea genotypes. Wroth (1998a, b) also used in situ inoculations to screen progeny families for their resistance to *M. pinodes*, and to study variation in pathogenicity among and within *M. pinodes* populations.

To date, no study has specifically focused on factors affecting the expression of partial resistance to ascochyta blight in pea. We therefore carried out experiments to identify which factors influence the expression of partial resistance to *M. pinodes* and *P. medicaginis* var *pinodella* in pea, and to determine optimum screening conditions to achieve maximum levels of differentiation among pea genotypes. We focused our study on two main components of partial resistance which are key factors in disease expression, namely fleck coalescence and lesion expansion. Fleck coalescence takes into account the early stages of interaction, from the inoculation to the first typical necrotic symptom, corresponding to the hemibio-trophic phase of the pathogen (Clulow et al. 1991), where different mechanisms of resistance are involved (Wroth 1998a). Lesion expansion reflects the growth rate of the pathogen in the host during the necrotrophic phase (Parlevliet 1979). A set of six genotypes differing in their levels of susceptibility to *M. pinodes* and *P. medicaginis* var. *pinodella* as determined by Onfroy et al. (1999), was used to define the effects of spore concentrations, fungal colony age prior to harvest of spores, and pathogenicity of isolates on these components of partial resistance assessed on detached leaves. As a result, a protocol is proposed for a reliable screening test to identify and quantify partial resistance to ascochyta blight in pea.

Materials and methods

Plant material

A set of six genotypes differing in their levels of susceptibility to *M. pinodes* and *P. medicaginis* var. *pinodella* (Onfroy et al. 1999) were used to test the effect of different factors on the expression of resistance. The germplasm line DP and the breeding

line FP (synonym CE101, Baranger et al. 2004) were defined as having a high level of partial resistance. The cv. Melrose was defined as partially resistant, the germplasm line JI 252 and the field pea cv. Solara (afila type) were found to be moderately susceptible, and the line JI 296 (garden pea cv. Chemin long) was highly susceptible. Seven other genotypes were included to study the correlation between disease reaction on detached stipules and seedlings, chosen on the basis of screening results for plantlet or adult plant resistance (Onfroy, unpublished results; Baranger, unpublished results): breeding lines CP and GP (synonym CF100, Baranger et al. 2004), germplasm lines JI96, GSP935 (PI288025) and GSP940a (PI343292), and winter pea cvs Champagne and Froidure. Origin and morphology data for all genotypes are described in Baranger et al. (2004), except for GSP935 (PI288025) and GSP940a (PI343292), which are described on the Pullman genebank website (http://www.ars-grin.gov). Three seeds of each genotype were planted in 9 cm diam pots containing a mixture of unsterilised soil/sand/peat (1:1:1). The soil originated from an experimental plot at the INRA research centre in Le Rheu. Six plants were used per genotype for the detached stipule assays. The pots were placed in trays in a growth chamber with a temperature of 15°C night/18°C day and a 14 h photoperiod with a light intensity of $160 \pm 2~\mu Em^{-2}s^{-1}$, until the plants reached the 5–6 leaf stage. For the seedling test, plant preparation and experimental design were carried out according to Onfroy et al. (1999).

Production of inoculum

Three *M. pinodes* isolates (Mp1, Mp2, Mp3), originating from different regions in France (Midi-Pyrénées, Normandy, Champagne), were compared for their effect on resistance expression to a *P. medicaginis* var. *pinodella* isolate (Pm1) originating from the central region of France. Subcultures of the isolates were taken from malt agar slants and grown on V8 medium (99 ml V8 vegetable juice (Campbell, France), 35 g agar, 801 ml distilled water, autoclaved at 105°C for 30 min) under white light with a 12 h photoperiod at 20°C (wavelengths between 350 and 750 nm). Pycnidiospore suspensions were prepared by flooding the surface of 10 day-old cultures with sterile distilled water, gently scraping the colony with

a glass rod and filtering the suspension through two layers of sterile cheesecloth (except for the experiment testing the age of the spores where 7-, 10- and 14 day-old cultures were used). The concentration of spores was determined with a haemocytometer and was adjusted to the required spore concentration (100, 500, 1000 and 5000 10 μl^{-1}). Tween 20 (VWR International SAS, Strasbourg, France) was added as a wetting agent (two drops 500 ml^{-1} spore suspension).

Inoculation and disease assessment on detached leaflets and stipules

The inoculation method used was based on that proposed by Heath and Wood (1969), consisting of depositing a drop of spore suspension on detached leaflets. Preliminary studies with the six genotypes used by Onfroy et al. (1999) revealed that (1) the reaction to ascochyta blight was identical on detached leaflets and on detached stipules, (2) the largest range between resistant and susceptible genotypes was observed on stipules from nodes 2, 3 or 4 of seedlings with 5–6 nodes (node 1 generally showed early senescence), and (3) a drop of 10 µl was optimal for inoculation (a drop of 5 µl evaporated too quickly, a drop of 20 µl induced lesions too large for accurate assessments). Short stem segments with attached stipules (referred to as detached stipules hereafter) from nodes 3 or 4 were used in all subsequent experiments because the cv. Solara is semi-leafless, and therefore lacks leaflets. After cutting, the detached stipules were floated, lower surface down, on tap water in a compartmented square Petri dish (12 cm side, Gosselin, France). Inoculation was with a drop of 10 µl of spore suspension placed on the upper surface of the stipules, avoiding the main veins. To avoid drop evaporation, Petri dishes were placed into large transparent plastic boxes.

From the six plants per genotype, two stipules were detached and inoculated each with a drop of the spore suspension resulting in 12 replicate assessments for each genotype. Detached stipules were incubated in a climatic chamber for an initial period of 18 h in the dark, followed subsequently by 7 days with a continuous cycle of 14 h light and 10 h darkness at 20°C. Symptom appearance on detached stipules was assessed each day after inoculation (dai) using a 0–3 semi-quantitative scale (fleck coalescence scale):

0 = symptom-free; 1 = flecks appearing; 2 = flecks covering half of the area of drop deposition; 3 = coalescence of the flecks within the area of drop deposition (approx. 3 mm).

Once necrosis had developed beyond the borders of each drop deposit, disease progress was assessed by measuring lesion diameter (mm) daily, with a graduated ruler, and was summarized as Area Under the Disease Progression Curve (AUDPC) calculated by plotting mean disease expansion against time according to the formulae proposed by Shaner and Finney (1977). In addition, the 0–7 scale based on different types of symptoms as described by Wroth (1998a) was adapted to our experimental conditions on detached stipules: 0 = symptom-free; 1 = flecks appearing; 2 = flecks covering half of the drop deposit; 3 = coalescence of the flecks in the area of the drop deposit (approx. 3 mm diam); 4 = 3–6 mm lesion diam; 5 = 6–9 mm lesion diam; 6 = 9–12 mm lesion diam, 7 = superior to 12mn lesion diam.

Inoculation and disease assessment on plantlets

Inoculation of seedlings by spraying spore suspensions of *M. pinodes* or *P. medicaginis* var *pinodella* was conducted as described by Onfroy et al. (1999). A spore suspension of 10^5 spores ml^{-1} was applied to plants at the 4–5 leaf stage using a hand-held garden sprayer and plants were incubated under a continuous cycle of 14 h at 18°C in light and 10 h at 15°C in darkness. Disease severity was assessed daily after inoculation using a 0–5 disease scale described previously (Onfroy et al. 1999). AUDPC was calculated using the formula proposed by Shaner and Finney (1977).

Data analysis

The effect of various factors on fleck coalescence and lesion expansion (including AUDPC) were analysed by ANOVA using the General Linear Model (GLM) procedure of the statistical package SAS version 8.1 (SAS 1988). The Student Newman-Keul's test ($P = 0.05$) was used to determine whether differences between plant genotypes, between fungal species or between isolates were statistically significant. Relationships between scoring criteria were tested by Pearson correlation analysis (SAS 1988).

Results

Effect of spore concentrations

The effect of inoculum pressure on partial resistance expression was investigated by inoculating detached stipules with different numbers of spores per drop: 100, 500, 1000, and 5000 (Fig. 1; Table 1). This experiment showed that, as expected, a drop containing 100 spores induced a slow appearance of symptoms. Two dai, the first flecks appeared only in the most susceptible genotypes JI296 and Solara. On the other hand, a drop containing 5000 spores induced a very fast development of symptoms. Disease severity was already very high at two dai and the area covered by the inoculation drop of all the genotypes was almost entirely necrotic, and no differences among the genotypes could be discerned. Concentrations of 500 and 1000 spores drop^{-1} allowed differences between genotypes to be distinguished based on their partial resistance (Fig. 1). Expanding lesions were first observed on genotypes JI 296, Solara and JI252 for 100, 500 and 1000 spores drop^{-1}. With 100 spores drop^{-1}, only the genotypes Solara (at 5 and 7 dai) and JI296 (at 7 dai) reached the lesion expansion phase. On the other hand, a dose of 5000 spores drop^{-1} differentiated susceptible and
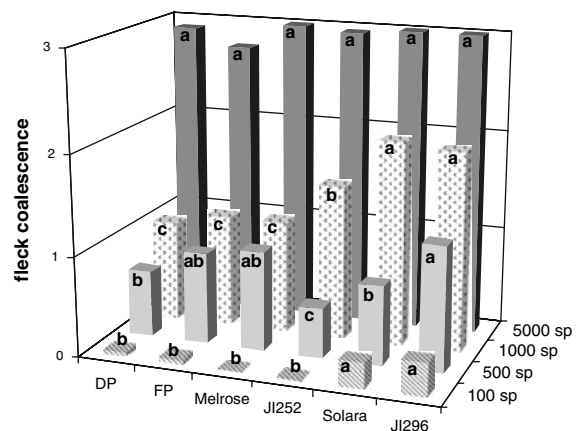
Fig. 1 Mean fleck coalescence scores (scale 0–3) on detached stipules of a set of six pea genotypes, 2 days after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at four concentrations. For each spore concentration (sp), fleck coalescence means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

Table 1 Mean lesion diameters (mm) on detached stipules of a set of six pea genotypes at 3, 5 and 7 days after point inoculation (dai) with spore suspensions of *Mycosphaerella pinodes* isolate (Mp1) at four concentrations

No. spores drop^{-1}	dai	Genotypes					
		DP	FP	Melrose	JI252	Solara	JI296
100	3	*fc*	*fc*	*fc*	*fc*	*fc*	*fc*
	5	*fc*	*fc*	*fc*	*fc*	4.7	fc
	7	*fc*	*fc*	*fc*	*fc*	9.3	8.9
500	3	*fc*	*fc*	*fc*	*fc*	*fc*	*fc*
	5	*fc*	*fc*	*fc*	3.9	6.2	5.8
	7	6.2 c	4.2 d	5.9 c	7.0 c	10.5 b	15.3 a
1000	3	*fc*	*fc*	*fc*	*fc*	3.1	3.1
	5	5.6 b	4.5 c	5.6 b	5.9 b	7.0 a	6.9 a
	7	10.0	*owa*	10.0	*owa*	11.6	16.1
5000	3	4.4 d	3.0 e	5.4 c	7.2 a	6.7 b	7.2 a
	5	*owa*	6.6 ± 0.7	*owa*	*owa*	10.9	11.3
	7	*owa*	*owa*	*owa*	*owa*	16.8	18.3

fc = fleck coalescence; *owa* = necrosis spreading over whole area of the stipule

For each spore concentration × dai combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

resistant genotypes only at 3 dai, whereas longer periods of incubation led to the rapid development of necrosis on the stipule surfaces. With 500 and 1000 spores drop^{-1}, lesion diameters discriminated better between genotypes and were significantly larger in genotypes JI 296 and Solara, and significantly smaller in genotype FP (Table 1). Strong effects of spore concentrations were observed both on fleck coalescence and lesion expansion. Concentrations too low (drops containing 100 spores) or too high (drops of 5000 spores) were inadequate for monitoring any component of resistance. Drops containing 500 or 1000 spores were more likely to reveal a range of partial resistance of both components. With drops containing 500 spores, the standard deviations were greater than with drops containing 1000 spores both for fleck coalescence and lesion expansion.

A further experiment was carried out, consisting of daily assessments of lesion diameters from 2 to 7 dai on stipules inoculated with 500 or 1000 spores drop^{-1} (Fig. 2). Because of the small size of its stipules, lesion diameters on genotype JI252 were measured only up to 5 dai. Differences between susceptible and resistant genotypes were mainly due to a delay in the onset of lesion expansion (3 or 4 dai depending on the genotype), whereas the slopes of plots of lesion expansion (i.e., increase in diameter) against time

were similar for the all six genotypes tested ($P > 0.05$).

AUDPC based on increases in lesion diameter from 4 to 7 dai, revealed significant differences among the five genotypes (Table 2). Lesion diameters assessed 5 dai allowed for comparisons between the six genotypes including JI252. The results showed that both spore concentrations were adequate in revealing differences in partial resistance of genotypes FP and DP. Genotypes Solara and JI 296 were highly susceptible, while genotypes Melrose and JI252 showed an intermediate reaction. A concentration of 500 spores drop^{-1} allowed slightly better discrimination within these intermediate genotypes than 1000 spores, indicating that JI252 is more resistant than Melrose.

Effect of fungal colony age on the pathogenicity of spores and expression of partial resistance

This experiment aimed at assessing the effect of the age (7, 10 or 14 day-old) of colonies from which spores for inoculation were harvested, on the expression of partial resistance on detached stipules. Spores harvested from a 7 day-old colony were significantly more aggressive than spores from older cultures,

Fig. 2 Disease progress curves based on mean lesion diameters on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at inoculum concentrations of (**a**) 500 spores and (**b**) 1000 spores drop^{-1}. Sp: spores
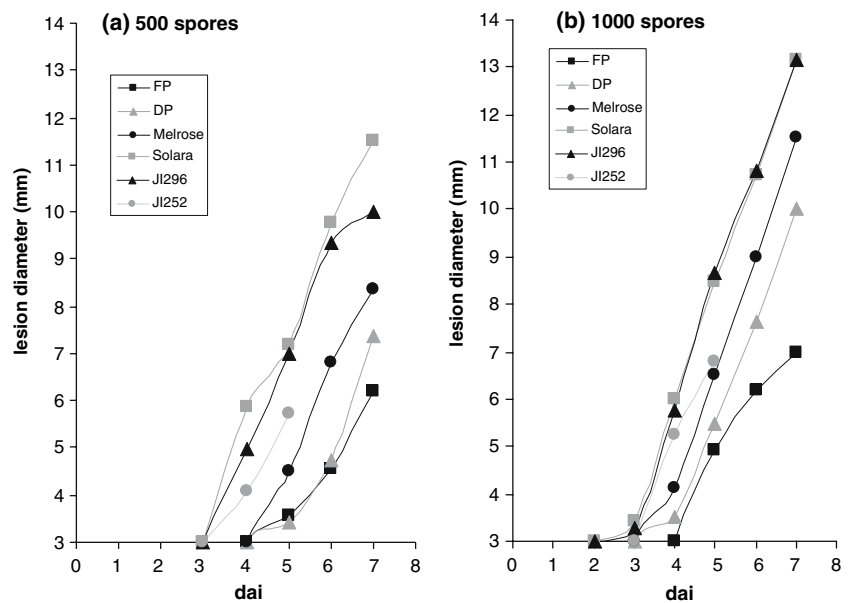
Table 2 Mean lesion diameters (mm) at 5 days after inoculation (dai) and AUDPC calculated from increasing lesion diameters from 4 to 7 dai on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate (Mp1) at two concentrations

	No. spores drop^{-1}	Genotypes					
		DP	FP	Melrose	JI252	Solara	JI296
Lesion diameter	500	3.4 d	3.5 d	4.5 c	5.8 b	7.2 a	7.0 a
	1000	5.5 c	4.9 c	6.5 b	6.8 b	8.5 a	8.7 a
AUDPC	500	4.4 d	3.7 d	8.0 c	–	16.6 a	14.8 b
	1000	10.8 c	7.1 d	14.3 b	–	19.8 a	20.0 a

For each spore concentration (i.e., for each line of the table), lesion diameter and AUDPC means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

irrespective of spore concentration (Table 3). For example, the average fleck coalescence scores for the six genotypes 2 dai were 1.3, 0.8 and 0.7 for spores obtained from 7, 10 and 14 day-old colonies, respectively, when inoculated at 500 spores drop^{-1}. Extensive lesions in the most susceptible genotypes were already observed at 3 dai when using inoculum from 7 day-old colonies (genotype JI296), whereas inoculum from 10 and 14 day-old colonies allowed data to be obtained for all genotypes both at 2 and 3 dai. Furthermore, ranges for partial resistance and differentiation among genotypes were best for inoculum from 10 and 14 day-old colonies. At 1000 spores drop^{-1}, fleck coalescence and expansion of lesions occurred more rapidly and data could be obtained for all genotypes only at 2 dai. Differentiation

among genotypes was not as accurate as with a drop containing 500 spores.

A very clear effect of colony age was also observed for lesion expansion over time, summarized as AUDPC. Average AUDPC was significantly higher for inoculum from 7 day-old colonies than from 10 or 14 day-old colonies (Table 3). Thus, for drops containing 500 spores, lesion diameter mean values for AUDPC over all genotypes were 8.4, 5.3 and 5.7, respectively, and for drops containing 1000 spores, these values were 12.4, 10.0 and 9.9 for inoculum from 7, 10 and 14 day-old colonies, respectively (data not shown). Irrespective of colony age, differences among genotypes with regard to partial resistance were observed, but the expression of partial resistance was better displayed with spores

Table 3 Mean fleck coalescence scores for detached stipules of a set of six pea genotypes at 2 and 3 days after point inoculation (dai) with two concentrations of spore suspension of *Mycosphaerella pinodes* isolate (Mp1) harvested from 7, 10 and 14 day-old colonies

No. spores drop^{-1}	Age of the colony (days)	dai	Genotypes						
			DP	FP	Melrose	JI252	Solara	JI296	Overall mean
500	7	2	1.0 bc	0.8 c	1.0 bc	1.8 a	1.4 b	1.9 a	1.3 A
		3	1.4	1.7	1.3	2.9	3.0	*le*	
	10	2	0.3 c	0.3 c	0.4 c	0.8 bc	1.1 b	1.8 a	0.8 B
		3	1.0 c	1.2 c	1.0 c	1.8 b	2.6 a	3.0 a	
	14	2	0.0 c	0.2 c	0.8 b	0.8 ab	1.0 ab	1.3 a	0.7 B
		3	1.0 c	1.0 c	1.0 c	2.4 b	2.7 b	3.0 a	
1000	7	2	2.0 bc	1.8 c	2.0 bc	2.6 ab	2.3 abc	2.8 a	2.3 A
		3	2.9	3.0	3.0	*le*	*le*	*le*	
	10	2	1.0 b	0.9 b	1.1 b	1.7 a	2.1 a	2.1 a	1.5 B
		3	2.0	2.3	2.0	3.0	3.0	*le*	
	14	2	0.8 b	0.8 b	1.0 b	1.8 a	1.7 a	1.7 a	1.3 C
		3	1.9	2.4	2.1	3.0	3.0	*le*	

le = lesion expansion

For each spore concentration × age of the colony combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; SNK test ($P = 0.05$)

For each spore concentration, lesion diameter means over all genotypes (i.e., for the last column of the table) for each age of the colony showing the same upper case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

from 10 and 14 day-old colonies (Fig. 3). For instance, mean lesion diameter values for AUDPC for genotype DP using drops containing 1000 spores were 4.5 and 4.3 for spores harvested from 10 and 14 day-old colonies, but had already reached 8.6 for spores obtained from 7 day-old colonies. Furthermore, results from this experiment indicate that the expression of partial resistance in the genotype JI252 collapsed with drops containing 1000 spores.

Effect of the isolate

Three *M. pinodes* and one *P. medicaginis* var. *pinodella* isolates were considered for their effects on the expression of partial resistance. At 2 dai, significant differences in fleck coalescence were observed between isolates (Table 4). The *P. medicaginis* var. *pinodella* isolate was generally far less aggressive than the *M. pinodes* isolates. Significant differences in fleck coalescence were also observed among the three *M. pinodes* isolates, with Mp1 and Mp2 being the least and Mp3 the most aggressive isolate. Although the disease symptoms appeared later with the *P. medicaginis* var. *pinodella* isolate, it

was still possible to discern significant differences between resistant and susceptible genotypes 2 dai with drops containing 1000 spores. Irrespective of the *M. pinodes* isolate and inoculum concentration, differences among genotypes could only be observed at 2 dai, since at 3 dai the most susceptible genotypes had always reached a mean fleck coalescence of 3.

AUDPC calculated from lesion diameters between 3 and 6 dai confirmed significant differences in pathogenicity among *M. pinodes*, and between *M. pinodes* and *P. medicaginis* var. *pinodella* isolates (Fig. 4). Thus, inoculations with Mp1, Mp2, Mp3 and Pm1 resulted in AUDPC means of all genotypes of 6.2, 6.5, 9.0 and 2.1, respectively, for drops containing 500 spores, and 9.2, 11.3, 13.7 and 5.6, respectively, for drops containing 1000 spores (data not shown). Furthermore, statistically significant differences between susceptible and resistant genotypes were displayed irrespective of the *M. pinodes* isolate and spore concentrations (Fig. 4). For the *P. medicaginis* var. *pinodella* isolate, differences between genotypes were best displayed with drops containing 1000 spores. No specific effect of any *M. pinodes* isolate was observed on disease progress (data not
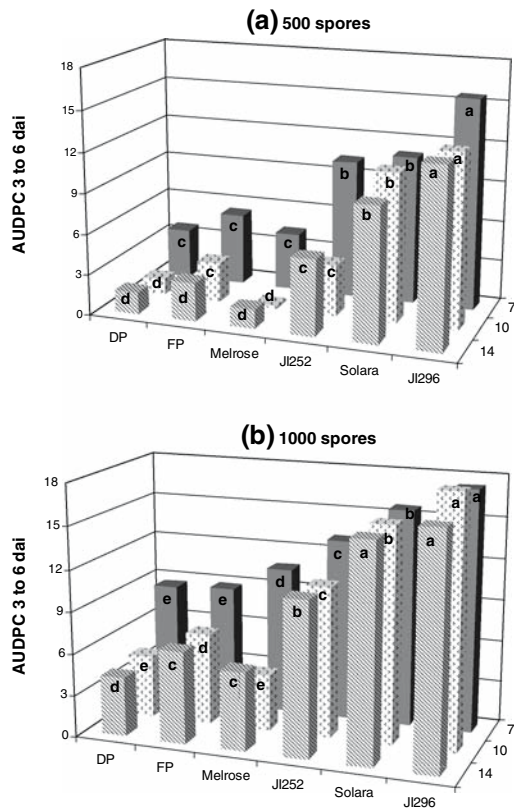
Fig. 3 Mean AUDPC calculated from lesion diameters from 3 to 6 days on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at inoculum concentrations of (**a**) 500 spores and (**b**) 1000 spores drop^{-1}, from 7, 10 and 14 day-old colonies. For each age of the colony, AUDPC means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

shown). However, a combination of a highly aggressive isolate (such as Mp3) and a high spore concentration did not allow differences in fleck coalescence to be observed among genotypes. Therefore the choice of a moderately aggressive *M. pinodes* isolate (such as Mp1) may allow discrimination between genotypes under a wider range of conditions.

Validating of conditions using an enlarged set of genotypes

We tested the conditions identified above for screening for partial resistance to *M. pinodes* on detached stipules (stipule or leaflet from node 3 or 4, drop size of 10 μl with 500 or 1000 spores obtained from colonies of 10–14 days, isolate moderately aggressive)

using an enlarged set of 13 genotypes. Fleck coalescence (Table 5A) covered a rather large range both at concentrations of 500 spores drop^{-1} (from 0.5 to 1.9 at 2 dai, and from 1.3 to 3.0 at 3 dai) and of 1000 spores (from 1.0 to 3.0 at 2 dai). With 500 spores per drop, three distinct groups of genotypes could be distinguished at 3 dai, one with the most resistant genotypes (FP, GP and Champagne), one with the most susceptible genotypes (Solara, CP, JI96, JI296, 935 and JI252), and an intermediate group with moderately susceptible genotypes, including DP, 940a, Melrose, and Froidure). When inoculated with 1000 spores drop^{-1}, these groups could not be separated as easily 2 dai as was possible after inoculation with a lower concentration of spores. However, overall, the same genotype classification was observed for both inoculum concentrations.

AUDPC calculated from lesion diameters between 3 and 6 dai also showed differences between genotypes (Fig. 5A). Genotype groupings were consistent with those based on fleck coalescence. Genotypes showing a delay in fleck coalescence also displayed the lowest AUDPC. Correlation coefficients between both components of resistance (fleck coalescence and AUDPC based on lesion expansion) were highly significant. At 500 spores drop^{-1}, R^2 values were 0.73 and 0.89 at 2 dai and 3 dai, respectively, whereas at 1000 spores drop^{-1}, R^2 values were 0.83 and 0.77 at 2 dai and 3 dai, respectively.

Comparison between detached stipules and the seedling tests

To check if partial resistance observed on detached stipules was correlated with partial resistance displayed in a seedling test, the results obtained from both methods were compared for this enlarged set of 13 genotypes (Fig. 5B). On seedlings inoculated with a spore suspension of 10^5 spores ml^{-1}, AUDPC was calculated based on disease severity measured between 4 and 11 dai (Fig. 5B). The mean AUDPC values showed a large range among genotypes, from 15.7 to 34.7 for lines FP and JI296, respectively. Mean AUDPC on seedlings was significantly correlated to fleck coalescence on detached stipules (R^2 ranging from 0.65 to 0.79) depending on spore concentration × dai combination, and to AUDPC based on lesion expansion on detached stipules

Table 4 Mean fleck coalescence scores for detached stipules of a set of six pea genotypes at 2 and 3 days after point inoculation (dai) with spore suspensions of three isolates of *Mycosphaerella pinodes* (Mp1–3) and one of *Phoma medicaginis* var. *pinodella* (Pm1) at two concentrations

No. spores drop^{-1}	Isolate	dai	Genotypes						
			DP	FP	Melrose	JI252	Solara	JI296	Overall mean
500	Mp 1	2	1.0 b	1.2 b	0.9 b	0.8 b	1.8 a	2.0 a	1.3 B
		3	1.6 b	2.3 ab	2.0 b	2.8 a	*le*	*le*	
	Mp2	2	0.8 b	1.3 b	1.0 b	1.0 b	1.8 a	1.9 a	1.3 B
		3	1.9 b	2.8 a	1.9 b	2.6 a	*le*	*le*	
	Mp 3	2	1.0 b	1.7 a	1.0 b	1.5 a	2.0 a	1.8 a	1.5 A
		3	2.7	*le*	2.3	*le*	*le*	*le*	
	Pm 1	2	0.3 b	0.2 b	0.4 a	0.1 b	0.8 a	0.2 b	0.3 C
		3	0.8 b	0.7 b	0.7 b	0.9 b	1.4 b	3.0 a	
1000	Mp 1	2	1.9 a	1.8 a	1.5 b	1.1 c	2.0 a	2.0 a	1.7 B
		3	3.0 a	3.0 a	3.0 a	3.0 a	*le*	*le*	
	Mp2	2	2.0 a	1.4 b	2.0 a	2.0 a	2.0 a	2.0 a	1.9 A
		3	3.0 a	3.0 a	3.0 a	3.0 a	*le*	*le*	
	Mp 3	2	2.0 a	2.0 a	2.0 a	2.0 a	2.0 a	2.0 a	2.0 A
		3	3.0 a	*le*	3.0 a	*le*	*le*	*le*	
	Pm 1	2	1.0 bc	0.7 c	1.0 bc	0.9 bc	1.8 a	1.4 b	1.1 C
		3	1.1 c	1.2 c	1.1 c	2.2 b	2.8 a	*le*	

le = lesion expansion

For each spore concentration × fungal isolate combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

For each spore concentration, lesion diameter means over all genotypes (i.e., for the last column of the table) for each fungal isolate showing the same upper case letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

($R^2 = 0.74$ for drops of 500 spores and $R^2 = 0.75$ for drops of 1000 spores).

Assessment of a scale combining both resistance components

To potentially simplify screening procedures, we additionally assessed the data with a scale including both resistance components. Mean AUDPC values based on this scale and assessments from 2 to 6 dai ranged from 9.8 to 15.8 with inoculum of 500 spores drop^{-1}, and from 12.0 to 17.5 with 1000 spores drop^{-1}, and displayed expected groupings among genotypes (Table 5B). Significant correlations were observed between AUDPC assessed on whole seedlings (data from Fig. 5), and AUDPC values from detached stipules inoculated with drops containing 500 spores ($R^2 = 0.81$) and with drops containing 1000 spores ($R^2 = 0.79$) after assessment with this modified scale.

Discussion

Expression of partial resistance

The results obtained in this study show that partial resistance of pea to *M. pinodes* is expressed and can be assessed on detached stipules in the form of two important epidemiological components: fleck coalescence and lesion expansion. In our experiments, the genotype DP reduced fleck coalescence, but showed lesion expansion similar to susceptible genotypes. This suggests that these parameters are under different genetic controls. With another legume fungus, *B. fabae*, the same phenomenon was observed with *Vicia narbonensis* which considerably delayed the initial establishment of infection, but was unable to limit spread in the leaflet tissue (Tivoli et al. 1986). This indicates that there are two different components in host resistance to disease, affected by spore concentration, age of the fungal colony from which
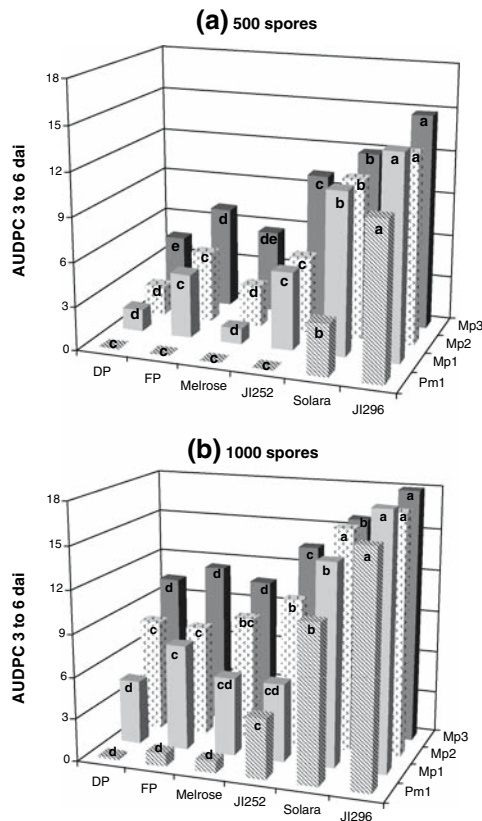
Fig. 4 Mean AUDPC calculated from lesion diameters from 3 to 6 days after inoculation on detached stipules of a set of six pea genotypes after point inoculation with spore suspensions of three isolates of *Mycosphaerella pinodes* (Mp1, Mp2 and Mp3) and one isolate of *Phoma medicaginis* var. *pinodella* (Pm1), at inoculum concentrations of (**a**) 500 spores and (**b**) 1000 spores drop^{-1}. For each fungal isolate, AUDPC means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test ($P = 0.05$)

spores are harvested, and isolate pathogenicity. Furthermore, we have shown that partial resistance can collapse when factors are too favourable for disease development, in this case when aggressive spores from a 7 day-old culture were used, a highly aggressive isolate was chosen and/or detached stipules were inoculated at a high spore concentration. This phenomenon was mainly observed with the line DP during lesion expansion. The effect of spore age on infection processes was described for *B. fabae* (Harrison 1988). Here, it was shown that infection hyphae from only young conidia may be able to kill host cells before appreciable phytoalexin synthesis has occurred. This observation suggests than the

same phenomenon could be involved in the case of *M. pinodes* and pea phytoalexins. The expression of partial resistance depends on parameters which are well defined, and its assessment is a compromise between disease expression and the expression of partial resistance. Our studies have also shown that each of the components of partial resistance assessed here was highly correlated with a seedling pathogenicity test.

Numerous factors may influence the expression of resistance. Biotic conditions that are best suited for pathogen development, high inoculum pressure and the use of highly aggressive strains are probably not suited for the identification of resistance components and partial resistance. We show that the best conditions to identify partial resistance are those with intermediate inoculum pressure, marginally favouring the pathogen. This idea was supported by Sakar et al. (1982) who showed that intermediate concentrations of *P. medicaginis* var. *pinodella* inoculum gave a better separation of mean foot-rot disease scores for three cultivars, compared to low or high concentrations. Results from our study suggest that high concentrations of inoculum make it more difficult to detect any differences among cultivars, whereas low concentrations can increase the variability in the data. Using similar approaches as described here, Wroth (1998a, b) studied resistance of host progenies and variation in pathogenicity among and within *M. pinodes* populations at two spore concentrations (500 and 1000 spores drop^{-1}). She observed a better discrimination among the breeding lines and a larger distribution pattern when leaves were inoculated with 500 spores, as well as a better characterisation of pathogen diversity at low inoculum pressure, mainly at day 10. Similar to results by Wroth (1998a, b), our results on the use of isolates with different levels of pathogenicity also lead to the following conclusions: to maximise the variation in host responses, it is better to use an aggressive isolate at low inoculum pressure (500 spores drop^{-1}) or a less aggressive isolate at high inoculum pressure (1000 spores drop^{-1}).

The observations we have made in this study are in agreement with the results obtained by Onfroy et al. (1999) and Prioul et al. (2003). The range between resistant and susceptible genotypes is the same as was observed by these authors. Based on 13 genotypes tested for the two components considered, this study

Table 5 Behaviour of a set of 13 pea genotypes after point inoculation of detached stipules with spore suspensions of *Mycosphaerella pinodes* isolate Mp1 at two concentrations expressed by; (A) Mean fleck coalescence scores at 2 and 3 dai and; (B) Mean AUDPC for lesion expansion assessed from 2 to 6 dai using a modified scale from Wroth (1998a)

No. spores drop⁻¹	dai	Genotypes													
		Champ	FP	GP	DP	Froidure	Melrose	935	JI252	940a	Solara	CP	JI96	JI296	Overall mean
(A)															
500	2	1.0 bc	0.5 d	0.9 c	1.0 bc	1.0 bc	1.0 bc	1.1 bc	1.4 b	1.1 bc	1.3 b	1.3 b	1.0 bc	1.9 a	1.1 B
	3	1.5 d	1.4 d	1.3 d	2.0 c	2.1 c	1.9 c	2.5 b	2.6 b	2.0 c	2.7 ab	2.9 ab	3.0 a	3.0 a	2.2 B
1000	2	1.6 d	1.0 c	1.0 e	1.9 cd	1.5 d	1.7 d	1.9 cd	1.9 cd	2.2 bc	2.3 b	1.9 cd	2.5 b	3.0 a	1.9 A
	3	2.4 b	2.1 c	2.0 c	2.9 a	2.8 a	2.7 a	3.0 a	3.0 a	3.0 a	3.0 a	3.0 a	3.0 a	3.0 a	2.8 A
(B)															
500		10.4 gh	9.8 h	10.6 fgh	11.5 efg	11.8 de	10.6 fgh	12.8 d	12.8 d	11.6 ef	13.9 c	15.3 ab	14.6 bc	15.8 a	12.4
1000		12.4 f	12.1 f	12.0 f	13.6 e	13.6 e	14.0 de	14.4 d	15.4 c	15.3 c	16.0 bc	16.0 bc	16.6 b	17.5 a	14.5

le = lesion expansion; Champ = cv. Champagne

For each spore concentration × dai combination (i.e., for each line of the table), lesion diameter means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test (*P* = 0.05)

For each spore concentration, lesion diameter means over all genotypes (i.e., for the last column of the table) showing the same upper case letter are not significantly different; Student Newman-Keul's test (*P* = 0.05)

For each spore concentration (i.e., for each line of the table), AUDPC means of genotypes showing the same lower case letter are not significantly different; Student Newman-Keul's test (*P* = 0.05)

Fig. 5 Behaviour of a set of 13 pea genotypes with spore suspensions of *Mycosphaerella pinodes* isolate Mp1; (**A**) after point inoculation on detached stipules at inoculum concentrations of (a) 500 spores and (b) 1000 spores drop^{-1}, expressed by mean AUDPC calculated from lesion diameters from 3 to 6 dai; and (**B**) after spraying on seedlings at 10^{5} spores ml^{-1}. Expressed by mean AUDPC calculated from disease severity assessed from 4 to 11 dai. AUDPC means of genotypes showing the same letter are not significantly different; Student Newman-Keul's test (*P* = 0.05)
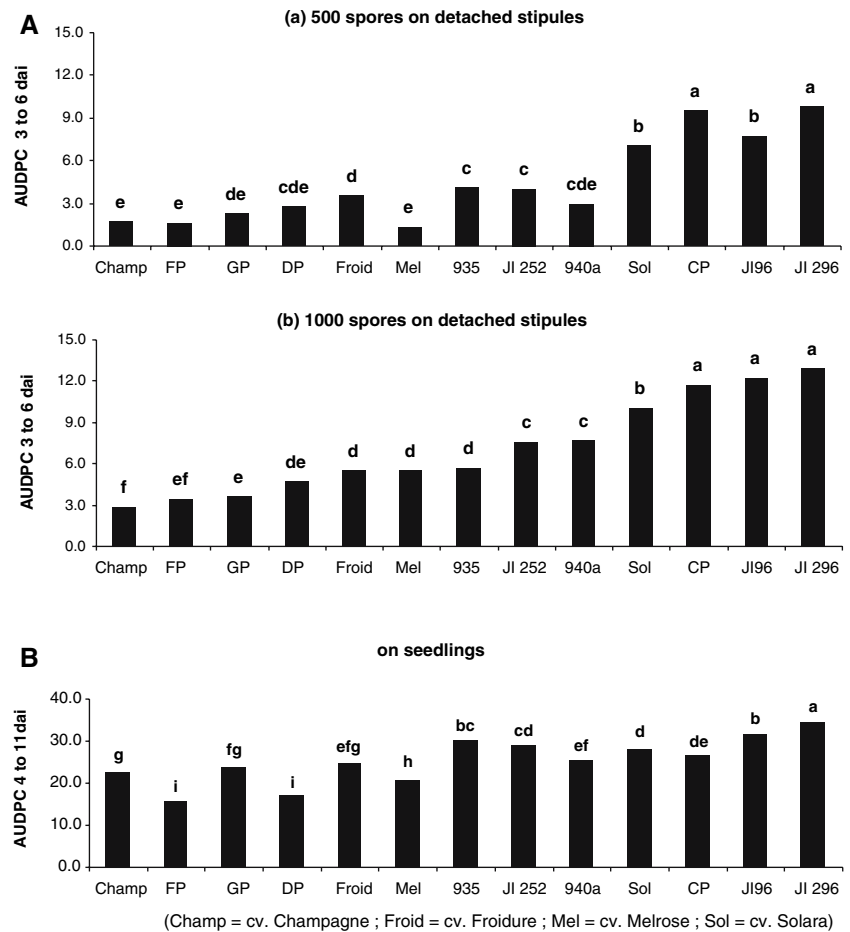
has demonstrated that the difference between resistant and susceptible genotypes is best determined using fleck coalescence rather than on the rate of subsequent lesion expansion, which is the same for resistant or susceptible genotypes. In addition, we confirmed that in spite of the weak pathogenicity of *P. medicaginis* var. *pinodella*, the range of resistance expression is the same for *M. pinodes* and *P. medicaginis* var. *pinodella*. Partial resistance does not appear to be species-specific between these two very close species of the ascochyta complex. The mechanisms of resistance to both pathogens could therefore be the same.

Methodology of screening

An understanding of the parameters that determine ideal conditions for the precise assessment of partial resistance among host genotypes is of crucial importance for the establishment of standardised environmental and inoculation conditions. Under such conditions, specific methodologies can be developed to assess the disease. Inoculum concentration, inoculum age, growth conditions of plants and plant phenology should be taken into account when determining components of resistance (Parlevliet 1979) and studying the conditions under which resistance is expressed. In our environmental conditions, the best conditions we have established to display partial resistance to *M. pinodes* on detached stipules of pea are: stipule or leaflet from node 3 or 4, drop size of 10 μl with 500 or 1000 spores harvested from a colony of 10–14 days, and use of a moderately aggressive isolate. The disease scale based on that by Wroth (1998a), which takes into account both components of resistance together (fleck coalescence and disease expansion), simplifies disease assessment and permits studies of a large number of host

genotypes. The strong correlation we obtained between the seedling test and the test on detached organs, which has also been observed by Dolar et al. (1994) on chickpea and Hwang et al. (2006) on pea inoculated with the respective ascochyta blight pathogens, strongly supports the feasibility of using detached leaf methods for resistance screening or other purposes. Both methodologies (seedling and detached stipule), address different resistance reactions. Spray inoculation of intact seedlings with spore suspensions, gives information on the overall behaviour of a genotype for its level of resistance whereas the detached stipule methodology is better suited for giving information on different components of resistance. Point inoculations of leaves have already been used for several objectives: to study resistance and/or components of resistance (Dolar et al. 1994; Bouhassan et al. 2003) and factors acting on phases of epidemic cycles (Heath and Wood 1969; Carisse and Peyrachon 1999), to characterise isolates for their pathogenicity/virulence (Nasir et al. 1992; Wroth 1998b) and to screen genotypes/lines for their resistance (Wroth 1999; Warkentin et al. 1995; Kohpina et al. 2000; Zhang et al. 2006).

The choice of method for scoring disease progress depends upon the objectives of the work. If the objective is to dissect partial resistance on a few host genotypes, both components of resistance, fleck coalescence and lesion diameter, can be used in routine screening, which were well correlated with a seedling test. A simplification of the method could be envisaged, consisting of an assessment of fleck coalescence at 2–3 dai, and lesion diameter at 5–6 dai (respectively for inoculum 1000 and 500 spores per 10 μl drop^{-1}). However, in some situations, earlier assessments better aligned to differentiate between different incubation times, may be more appropriate. For screening tests using hundreds of lines, it is likely to be more suitable to use the more comprehensive scale as described here, and modified from Wroth (1998a) as a first step, before dissecting specific components of resistance. Assessing disease with this scale at two dates will implicitly take into account both components of resistance, fleck coalescence and lesion expansion beyond the inoculation drop.

As shown by Bretag and Brouwer (1995) and Wroth and Khan (1999), it is difficult to evaluate partial resistance to ascochyta blight in the field, due

to factors interacting with disease severity assessments: agronomic traits (such as plant maturity, lodging, plant height and canopy architecture) or environmental conditions (such as climatic conditions and disease pressure levels). To obtain clearer insight into the main genetic effects involved in resistance, Prioul et al. (2003) and Hwang et al. (2006) tried to minimize these interactions by assessing resistance under controlled conditions. Fondevilla et al. (2005) and Hwang et al. (2006) have shown that cultivar rankings fluctuated across methodologies, but that ranking tended to be stable at the extremes (most resistant, most susceptible) between field and controlled conditions assessments. Likewise in most field trials, we observed significant differences between extreme genotypes DP and JI296 for their resistance to *M. pinodes* (data not shown). This methodology of detached stipules was used by Baranger et al. (2006) to develop further studies on genetic knowledge of resistance and QTL or gene identification. These authors have identified six QTL specifically involved in reducing *M. pinodes* fleck coalescence and lesion expansion.

We conclude that quantitative resistance can be expressed on detached pea stipules only under certain conditions, by expression on fleck coalescence and on lesion expansion. Other resistance components, mainly the reproduction of the pathogen (latent period, pycnidia/pseudothecial formation, number of spores), need to be studied. Reports show that often experimental conditions are the same to display different components of resistance. Vijanen-Rollinson et al. (1998) for instance, used the same conditions to study diverse components of quantitative resistance to powdery mildew in pea (conidial germination, infection efficiency, latent period and conidial production). Bouhassan et al. (2003) also analysed various components of partial resistance to chocolate spot in faba bean (incubation period, number of spots, lesion diameter, latency period and sporulation) under environmental conditions common to all components. The optimal experimental conditions we have defined for the expression of pea resistance to *M. pinodes* on fleck coalescence and lesion expansion might therefore be adapted to the study of other components of resistance. Further studies are needed to confirm this or show that some component evaluation would need specific environmental conditions. Furthermore, how these

components affect epidemic development on resistant genotypes in the field remains to be determined.

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FULL RESEARCH PAPER

Validation of a QTL for resistance to ascochyta blight linked to resistance to fusarium wilt race 5 in chickpea (*Cicer arietinum* L.)

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Abstract Ascochyta blight caused by *Ascochyta rabiei* and fusarium wilt caused by *Fusarium oxysporum*. f. sp. *ciceris* are the two most serious diseases of chickpea (*Cicer arietinum*). Quantitative trait loci (QTL) or genes for ascochyta blight resistance and a cluster of resistance genes for several fusarium wilt races (*foc1*, *foc3*, *foc4* and *foc5*) located on LG2 of the chickpea map have been reported independently. In order to validate these results and study the linkage relationship between the loci that confer resistance to blight and wilt, an intraspecific chickpea recombinant inbred lines (RIL) population that segregates for resistance to both diseases was studied. A new LG2 was established using sequence tagged microsatellite sites (STMS) markers selected from other chickpea maps. Resistance to race 5 of *F. oxysporum* (*foc5*) was inherited as a single gene and mapped to LG2, flanked by the STMS markers TA110 (6.5 cM apart) and TA59 (8.9 cM apart). A QTL for resistance to ascochyta blight (QTL$_{AR3}$) was also detected on LG2 using evaluation data obtained separately in two cropping seasons. This genomic region, where QTL$_{AR3}$ is located, was highly saturated with STMS markers. STMS TA194 appeared tightly linked to QTL$_{AR3}$ and was flanked by the STMS markers TR58 and TS82 (6.5 cM apart). The genetic distance between *foc5* and QTL$_{AR3}$ peak was around 24 cM including six markers within this interval. The markers linked to both loci could facilitate the pyramiding of resistance genes for both diseases through MAS.

Keywords *Cicer arietinum* · *Ascochyta rabiei* · *Fusarium oxysporum* · Molecular markers · Linkage analysis

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Introduction

Chickpea (*Cicer arietinum*) is an autogamous annual cool-season grain legume cultivated in arid and semi-arid areas across the six continents. It is valued for its high protein content and the absence of specific major anti-nutritional factors means that it is considered nutritional and healthy (Williams and Singh 1987; Gil et al. 1996). It is mostly used for human consumption and to a lesser extent for animal feed. Chickpea yield is

low and unstable with a global average below 0.8 t ha^{-1} (FAOSTAT 2005). Two fungal diseases, ascochyta blight (caused by *Ascochyta rabiei*; syn. *Phoma rabiei*) and fusarium wilt (caused by *Fusarium oxysporum* f. sp. *ciceris*), are important limiting factors for yield worldwide.

Ascochyta blight is the most destructive disease affecting chickpea in many farming regions of the world. *Ascochyta rabiei* can attack at any growth stage and affect all aerial parts of the plant, producing lesions with concentric rings of pycnidia and stem breakage due to girdling. Sources of resistance to ascochyta blight have been identified from *C. arietinum* and wild *Cicer* species (Singh and Reddy 1993; Collard et al. 2001; Chen et al. 2004). This resistance, available in cultivated chickpea, has been exploited in conventional breeding programmes, producing new resistant cultivars worldwide. Knowledge of the genetic bases of both virulence in *A. rabiei* and resistance in chickpea is essential in order to develop cultivars with more durable resistance. To date, the pathogen has been classified mainly into two broad pathotypes: pathotype I (less aggressive) and pathotype II (aggressive) (Chen et al. 2004); but further research is required to identify the genes that control aggressiveness. As for the host, early studies on the inheritance of blight resistance indicated that it could be conferred by one, two or three genes (Singh and Reddy 1983; Tewari and Pandey 1986; Dey and Singh 1993; Tekeoglu et al. 2000). Furthermore, evidence that resistance might be inherited as a quantitative trait has been reported (Muehlbauer and Kaiser, 1994). Resistance to blight is considered partial or incomplete in chickpea. Climatic conditions, inoculum density, pathotype variation and plant age all affect disease development. Thus, the use of RIL populations (homozygous lines) provides a more accurate evaluation of the disease than F$_2$ populations. Furthermore, RILs can be evaluated for reaction to different pathotypes and under differing environmental conditions. The use of RIL populations and molecular markers has contributed a great deal to the identification of quantitative trait loci (QTL) for resistance to ascochyta blight. Several QTL have been located on different maps developed by various authors and the STMS markers linked to

these QTL have helped to assign them to linkage groups relating to the most extensive chickpea map (Winter et al. 2000). Two major QTL (QTL-1 and QTL-2) that confer resistance have been located on linkage group 4 (LG4) by different authors (Santra et al. 2000; Tekeoglu et al. 2002; Collard et al. 2003; Flandez-Galvez et al. 2003; Millán et al. 2003; Rakshit et al. 2003; Udupa and Baum 2003; Cho et al. 2004; Iruela et al. 2006). We suggest labelling them QTL$_{AR1}$ and QTL$_{AR2}$ (Iruela et al., 2006). These two QTL seem to confer resistance to pathotype II of *A. rabiei* according to the results of Udupa and Baum (2003) and Cho et al. (2004). QTL$_{AR2}$ has been located in a genomic region with a high density of markers (Iruela et al. 2006) whereas QTL$_{AR1}$ appeared in a loose genomic region. Other genes or QTL for resistance to blight have been reported on LG2 in a poorly saturated region (Udupa and Baum 2003; Cho et al. 2004; Cobos et al. 2006) and seem to be more associated with pathotype I of *A. rabiei* (Udupa and Baum 2003; Cho et al. 2004).

Fusarium wilt is another serious disease that affects chickpea, decreasing production in many countries. Eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been reported. In susceptible chickpea cultivars, races 1A, 2, 3, 4, 5 and 6 induce the wilting syndrome, whereas races 0 and 1B/C induce the yellowing syndrome. Races 0, 1A, 1B/C, 5 and 6 are found mainly in the Mediterranean region and California (see review by Jiménez-Gasco et al. 2004). In Spain, race 5 is the most virulent (Landa et al. 2004) and along with race 6 is the second most common after race 0 (Jimenez-Diaz et al. 1989). Breeding programmes have been developed using resistant desi cultivars but the pathogenic variability of the fungus is an added difficulty. Studies using inter and intraspecific populations of chickpea and random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), sequence characterised amplified regions (SCAR) and sequence tagged microsatellite sites (STMS) markers indicated that resistance genes for fusarium wilt races 1, 3, 4 and 5 (*foc1*, *foc3*, *foc4* and *foc5*) are located on LG2, forming a cluster (Mayer et al. 1997; Ratnaparkhe et al. 1998a; Tullu et al. 1998; Winter et al. 2000; Sharma et al.

2004). Recently, one of the two genes that confers resistance to race 0 (Rubio et al. 2003) was mapped on LG5 (Cobos et al. 2005). However, the second gene for race 0 has been located on LG2 (unpublished data).

LG2 of the chickpea map is interesting because it contains resistance genes for fusarium wilt and QTL for ascochyta blight resistance, the two most important diseases worldwide. From the point of view of breeding, it is very important to know the linkage relationship (distance) between the QTL for resistance to blight and the resistance genes for fusarium wilt. This information could help to apply marker-assisted selection (MAS) for these two traits simultaneously, requiring a high number of tightly-linked markers flanking the QTL or genes. This study focused on a chickpea RIL population segregating for both diseases and mapped markers located on LG2, which enabled the linkage between the two diseases to be examined.

Materials and methods

Ascochyta blight and fusarium wilt resistance evaluations

A chickpea RIL population of 111 $F_{6:7}$ individuals derived from the intraspecific cross ILC3279 × WR315 was used. ILC3279 is a kabuli line from the former Soviet Union (maintained by the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria), which is resistant to ascochyta blight and susceptible to wilt. WR315 is a desi landrace from central India (maintained by the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT); Patancheru, India), which is resistant to all races of fusarium wilt and susceptible to blight. The single seed descent method was employed for RIL population development.

Ascochyta blight resistance reaction of RILs and parents were scored in field trials in 2002 and 2003 (Iruela et al. 2006). The RIL population was also evaluated for wilt reaction under controlled conditions in a growth chamber. Colonised filter paper cultures of *F. oxysporum* f. sp. *ciceris* race 5

(kindly provided by Dr. Muehlbauer, Washington State University, Pullman, USA) were cultured in potato-dextrose broth (24 g l^{-1}) at 25°C with light for one week to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheesecloth to remove mycelia. The spore suspension was then pelleted by centrifugation at low speed (3000 rpm) for 3 min. After the supernatant was discarded, the conidia were diluted with sterile water to obtain a concentration of 10^6 spores ml^{-1}. Parents and RILs seedlings at the three to four nodal stages were inoculated following the method described by Bhatti et al. (1990). The inoculated plants were grown in perlite in a growth room with a temperature regime of 25 and 22°C (12 h/12 h) under fluorescent light. The plants were watered daily and supplied with nutrient solution once a week after inoculation. Fusarium wilt incidence, scored as % of dead plants, was recorded 4 weeks after inoculation. RILs with 0–30% dead plants were considered resistant and RILs with 70–100% dead plants were considered susceptible.

Construction of molecular map and QTL analysis

The RIL population was genotyped for 10 STMS markers (GA16, TA37, TA53, TA59, TA103, TA110, TA194, TR19, TR58, TS82) and the SCAR marker CS27 selected from LG2 of both interspecific and intraspecific chickpea maps (Winter et al. 2000; Tekeoglu et al. 2002; Udupa and Baum 2003).

For DNA extraction, about 100 mg of young leaf tissue was excised, frozen immediately in liquid nitrogen and stored at –80° C. DNA was isolated using DNAZOL (Invitrogen). The STMS primer sequences and amplification conditions employed were described by Winter et al. (1999). The SCAR CS27, developed from the RAPD $CS27_{700}$ by Mayer et al. (1997), was analysed according to the protocol defined by these authors. Amplification products from STMS except TA37 were electrophoresed in 2.5% Metaphor agarose (Biowhitaker Molecular Application) gels. TA37 was analysed in 10% polyacrylamide gels and the SCAR CS27 in gels composed of a mixture of 1% SeaKem agarose

and 1% NuSieve agarose (Hispanlab SA). PCR fragments were stained with ethidium bromide.

Goodness of fit to the expected 1:1 segregation ratio of marker loci was tested using the χ^2 test. Linkage analysis was performed using JOINMAP 3.0 (Van Ooijen and Voorrips 2001) with a minimum LOD score of 3 and a maximum recombination fraction of 0.25. Kosambi's function was applied to estimate map distances in centiMorgans. MAPQTL 5 software (Van Ooijen, 2004) was employed to locate putative QTL for ascochyta blight resistance considering the disease evaluation data from the two cropping seasons. The interval mapping (IM) method with a mapping step size of 1 cM was applied, determining the significance thresholds for the LOD score through the permutation test (number of iterations = 1000, P = 0.05) (Churchill and Doerge 1994). The coefficient of determination (R^2) of the marker most closely linked to a QTL was used to estimate the percentage of the total phenotypic variation explained by the QTL.

Results

RIL population tested for reaction to wilt race 5 resulted in 50 resistant and 56 susceptible plants. This data fitted a 1:1 segregation ratio suggesting that a single gene controlled resistance to fusarium wilt race 5 (foc5) in this population. The resistant parental line (WR315) did not display symptoms of wilt and the susceptible parental line (ILC3279) had 100% dead plants.

The 10 STMS and the SCAR CS27, selected from previous chickpea maps because of their presence on LG2, revealed polymorphism between the parental lines and fitted the expected 1:1 ratio well when they were used to genotype the whole RIL population. As expected, all analysed markers and the locus foc5 formed a single linkage group (LG2) covering a genetic distance of 62 cM and showing a maximum and minimum distance between markers of 14.1 and 1.3 cM, respectively (Fig. 1). The resistance gene foc5 was flanked by the STMS markers TA110 (6.5 cM apart) and TA59 (8.9 cM apart). The SCAR CS27 was located 12.3 cM from this

resistance gene. The utilisation of locus-specific STMS markers meant that the LG2 obtained could be aligned with other LG2 previously reported in different populations. The order of the STMS markers on LG2 was identical to that found by Udupa and Baum (2003) and Tekeoglu et al. (2002), employing RIL populations derived from intra and interspecific crosses, respectively. Though the order of the markers was the same as that found by Tekeoglu et al. (2002), genetic distances between the STMS TA194 and TA53 were considerably different. TA53 was 4.8 cM compared to 80.8 cM apart in the LG2 reported by Tekeoglu et al. (2002). Difference in the order of the markers was observed when compared to the interspecific *Cicer* map of Winter et al. (2000). However, marker TA194 was tightly linked to TR58 and TS82 markers in both studies.

The AUDPC data obtained from the evaluations for ascochyta blight in each cropping season (Iruela et al. 2006) were considered separately. This disease reaction data was tested for associations with single markers contained on LG2. Five of them (GA16, TS82, TA194, TR58 and TA53), covering a map distance of 21.3 cM, were found to be significantly associated ($P < 0.001$) with resistance in 2002; in 2003, on the other hand, only one marker (TA194) was found to be associated. Interval mapping located a QTL for blight resistance (suggested name QTL_{AR3}) on this LG2 in both years (Fig. 2). This QTL had a maximum LOD value of 5.9 in 2002 and 2.5 in 2003 and significance level of 1.8 in both years. QTL_{AR3} explained 22.6% and 11.3% of the total phenotypic variation of blight reaction using 2002 and 2003 evaluation data, respectively. In both years, the QTL peak coincided with the position of STMS TA194, which was flanked by the STMS TR58 and TS82 (6.5 cM apart). The distance between TA194 and foc5 was around 24 cM.

Discussion

Microsatellite-based markers, such as STMS, have shown a significant degree of polymorphism in spite of the monotony of the chickpea genome, previously reported using isozymes, RFLP,

Fig. 1 Linkage group obtained in the chickpea RIL population ILC3279 × WR315 (black bar) and its alignment through common markers included in LG2 of Winter et al. (2000), Tekeoglu et al. (2002) and Udupa and Baum (2003). Map distances are in cM. Fusarium wilt race 5 resistant gene is in *bold*, the SCAR marker is *underlined* and the remainder of the markers are STMS
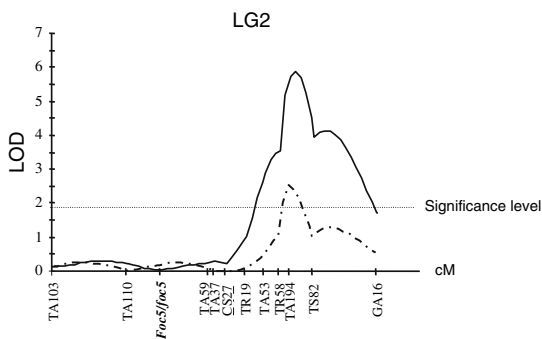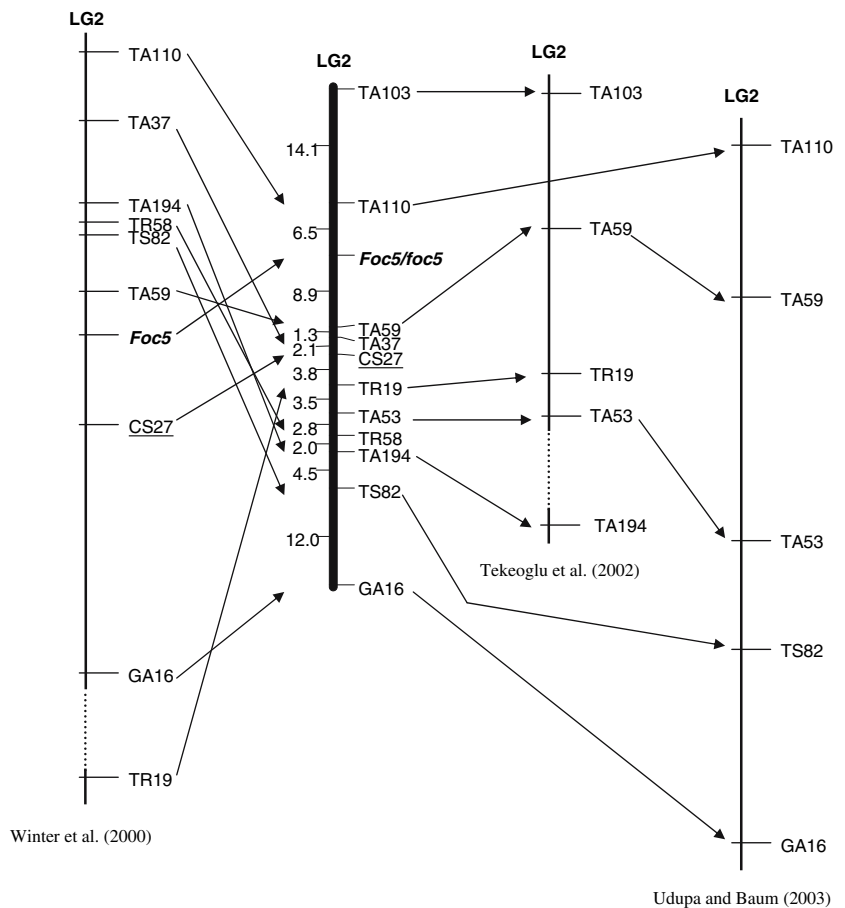
Fig. 2 QTL for ascochyta blight resistance obtained in the chickpea RIL population ILC3279 × WR315 evaluated in 2002 (—) and 2003 (–·–·). Fusarium wilt race 5 resistant gene is in *bold*, the SCAR marker is *underlined* and the remainder of the markers are STMS

RAPD, DAF and AFLP (see review by Winter et al. 2003). All STMS markers chosen from interspecific and intraspecific maps (Winter et al. 2000; Tekeoglu et al. 2002; Udupa and Baum 2003) were polymorphic, thus validating the potential of STMS in MAS. Differences in the order of markers compared with the linkage group of Winter et al. (2000) and a greater genetic distance between TA194 and TA53 in Tekeoglu et al. (2000) were observed. These may be due to the different origin of the RIL population used, which were derived from interspecific crosses in the case of Winter et al. (2000) and Tekeoglu et al. (2002), and intraspecific in this study. Furthermore, different software packages based on different procedures could affect the order or distance between markers. Population size is another factor to take into account; large populations would give a more accurate order of the markers. Consensus maps using different chickpea mapping populations that segregate for common markers across the populations, as reported in other crops (Doligez et al. 2006; Song

et al. 2004), could be one way of obtaining a more accurate chickpea map (Tekeoglu et al. 2002).

Resistance to race 5 of *F. oxysporum* was monogenic and was mapped to LG2, considering common STMS markers related to the reference chickpea genetic map of Winter et al. (2000). This result confirms previous reports of the monogenic nature of resistance to race 5 in WR315 (Sharma et al. 2005). Using another source of resistance, ICC4958, Tekeoglu et al. (2000) also demonstrated monogenic inheritance to race 5. This gene for resistance to race 5 present in ICC4958 was also located on LG2, linked to genes for resistance to races 1, 3 and 4 (Ratnaparkhe et al. 1998a, b; Tekeoglu et al. 2000; Winter et al. 2000). Genes for resistance to races 1, 3 and 4 present in WR315 have been also mapped to LG2 (indicative marker CS27) and could be considered to be the same as the one present in ICC4958 (Mayer et al. 1997; Tullu et al. 1998; Sharma et al. 2004). The gene conferring resistance to race 5 present in WR315 could be also considered the same as the one in ICC4958.

In addition to fusarium wilt resistance genes reported on LG2, genes or QTL associated with resistance to ascochyta blight were also found on this LG (Udupa and Baum 2003; Cho et al. 2004; Cobos et al. 2006). A major locus and a tightly linked QTL, which confer resistance to pathotype I and II respectively, were identified by Udupa and Baum (2003), who used the same resistant source employed in this study (ILC3279). Cho et al. (2004), using a different resistant parental line (FILP84-92C), also reported a major gene (Ar19) for resistance to pathotype I on LG2. In both studies, the genes or QTL were located in a poorly saturated genomic region and the closest marker was the STMS GA16 (around 20 cM apart). More recently, using an interspecific RIL population, another QTL for resistance to blight was located on LG2, flanked by a RAPD and a ISSR markers (14.1 cM apart) and the STMS TA103 was over 20 cM away from the peak of the QTL (Cobos et al. 2006). In this case, the resistance source was ILC72. Both markers flanked the QTL reported by Cobos et al. (2006) were monomorphic in the intraspecific population used in this study. There would need to be a higher density of markers around the genes in question

in order to know whether these genes or QTL are or not the same as those present in different parental lines, and also in order to use MAS for resistance. This study detected a QTL far away from STMS TA103 (>30 cM) and around 20 cM from GA16. This QTL might be the same as that reported by Cobos et al. (2006) (indicative marker TA103), and possibly the same as those reported by Udupa and Baum (2003) and Cho et al. (2004) (indicative marker GA16). STMS TA194, which was tightly linked to the QTL, was not present in the maps reported by the aforementioned authors. However, STMS markers flanking the QTL such as TA53 and TS82 were present in the map defined by Udupa and Baum (2003), but located more than 25 cM away from the gene for pathotype I (ar1) or the QTL for pathotype II (ar2a) of ascochyta blight. The latter were closer to GA16, located midway between TS82 and ar1 or ar2a.

As mentioned previously, the order of markers in a linkage group can be affected by different factors. Furthermore, experimental error in the disease score might have contributed to a different order. Udupa and Baum (2003) phenotyped the RILs for pathotype I on the basis of a bimodal rather than continuous distribution, where the score of the RILs with intermediate reactions might contribute to the experimental error. Cho et al. (2004) reported a major gene (Ar19) for pathotype I on LG2+6, mapped between TR19 and GA16. They suggested that Ar19 appeared to provide most of the quantitative resistance to pathotype I and, to a lesser extent, resistance to pathotype II. In a previous study, using the same RIL population as in the current study as well as the same scoring data obtained during 2002 and 2003, two strong QTL (QTL$_{AR1}$ and QTL$_{AR2}$) located on LG4 were reported in the second year only, suggesting that different pathotypes might be present in each of the evaluated years (Iruela et al. 2006). The QTL obtained on LG2 was more important in the first year and had only a slight presence in the second year. These results suggest that QTL$_{AR3}$ on LG2 could be the same as the QTL or genes for resistance to pathotype I of *A. rabiei* proposed by the cited authors. More work needs to be done to saturate the genomic region of LG2 where these genes or QTL have

been detected in order to get a more accurate validation. TA194 could be a good reference marker for verification.

In conclusion, this study has confirmed that the loci responsible for the two most economically important diseases of chickpea appear as a cluster on LG2. Complex clusters of disease resistance genes are common in plant genomes . Examples of *R* genes that are present in clusters include *Rp1, Rpp5, Xa21, Pto, Dm3, I2, N*, *M* and the *Cf* genes (Takken et al. 2000). In *Arabidopsis*, 109 of the 149 NB-LRR genes reside in 40 clusters ranging in size from two to eight genes, while the remaining 40 genes exist as singletons (Meyers et al. 2003). These clusters can span large chromosome segments and confer resistance to different races of the same pathogen as well as to different pathogens. For example, a common bean map revealed numerous resistance gene clusters, including the co-location of genes for resistance to two fungal diseases, anthracnose and rust (Miklas et al. 2006). Resistance genes to powdery mildew (*Rmd*-c), Phytophthora stem and root rot (*Rps*2), and an ineffective nodulation gene (*Rj*2) have been mapped within a cluster on linkage group J in soybean (Polzin et al. 1994). From the point of view of chickpea breeding, the genetic distance (around 24 cM) between both loci (*foc5* and TA194) do not appear to pose a problem for pyramiding resistance to fusarium wilt race 5 and the QTL$_{AR3}$ for ascochyta blight. The closely linked STMS markers to both loci could be used, via MAS, to achieve these objectives.

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Genetic relationships among Chickpea (*Cicer arietinum* L.) genotypes based on the SSRs at the quantitative trait Loci for resistance to Ascochyta Blight

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Abstract Breeding for resistance to ascochyta blight in chickpea has been challenged by several factors including the limited sources of good resistance. Characterization of a set of genotypes that may contain different genes for resistance may help breeders to develop better and more durable resistance compared to current cultivars. The objective of this study was to evaluate the genetic relationships of 37 chickpea germplasm accessions differing in reaction to ascochyta blight using Simple Sequence Repeat (SSR) markers linked to Quantitative Trait Loci (QTL) for resistance. The results demonstrated that ILC72 and ILC3279, landraces from the former Soviet Union, had SSR alleles that were common among the kabuli breeding lines and cultivars. A lower SSR allele diversity was found on LG4 than on other regions. No correlation was found between the dendrogram derived using SSRs at the QTL regions and the SSRs derived from other parts of the genome. The clustering based on 127 alleles of 17 SSRs associated with the QTL for ascochyta blight resistance enabled us to differentiate three major groups within the current germplasm accessions. The first group was the desi germplasm originating from

India and cultivars derived from it. The second group was a mix of desi genotypes originating from India and Greece, and kabuli breeding lines from ICARDA and the University of Saskatchewan. The third and largest group consisted of landraces originating mostly from the former Soviet Union and breeding lines/cultivars of the kabuli type. Several moderately resistance genotypes that are distantly related were identified. Disease evaluation on three test populations suggested that it is possible to enhance the level of resistance by crossing moderately resistant parents with distinct genetic backgrounds at the QTL for resistance to ascochyta blight.

Keywords Chickpea · Ascochyta blight resistance · Quantitative Trait Loci (QTL) · Simple Sequence Repeat (SSR)

Introduction

Ascochyta blight caused by the fungus *Ascochyta rabiei* is one of the most destructive diseases of chickpea worldwide resulting in reduced yield and quality. Yield losses of up to 100% have been reported in severely infected fields (Acikgoz et al. 1994). Seed treatment and foliar application of fungicides, as well as cultural practices are often unsuccessful and uneconomical for controlling this disease (Nene and Reddy 1987). The use of cultivars with high levels of resistance is considered the most

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economical solution for long-term disease management. The use of resistant cultivars will also help to stabilize chickpea production. Breeding for resistance to ascochyta blight in chickpea has been limited by several factors, including the high pathogenic variability of the fungus and the limited sources of good resistance (Khan et al. 1999; Singh and Reddy 1993).

Early studies by Singh and Reddy (1993) demonstrated that only five genotypes (ICC4475, ICC6328, ICC12004, ILC200 and ILC6482) out of 19,343 accessions were resistant to ascochyta blight in repeated field and greenhouse evaluations. Chen et al. (2004) further reported that the germplasm lines ICC3996, ICC4475 and ICC12004 were resistant against a number of A. rabiei isolates originating from northwestern United States. Several other accessions of different origins with reported resistance to ascochyta blight included: ILC72, ILC195, ILC200, ILC482, ILC3279 and ILC6482 (Reddy and Singh 1992; Singh et al. 1992; 1993). Most breeding programmes worldwide have relied heavily on two kabuli genotypes, ILC72 and ILC3279, as sources for ascochyta blight resistance (Crino 1990; Muehlbauer et al. 1998, 2004; Muehlbauer and Kaiser 2002; Millan et al. 2003; Rubio et al. 2004). In many cases, the occurrence of new pathotypes or the increased aggressiveness of the current A. rabiei pathotypes have broken the resistance in several of these varieties. For example, cv. Sanford was initially considered as a resistant variety; however, after several years of production under Saskatchewan environments it became very susceptible. The use of additional resistance sources in breeding programmes is needed to diversify the genetic basis of resistance in elite chickpea germplasm and/or to increase the level of resistance through gene pyramiding. It is crucial, therefore, to characterize accessions from diverse origins that may contain different genes for resistance to ascochyta blight. This will allow breeders to select sources of resistance that may contain different genes and to accumulate those genes in one cultivar to enhance the levels of resistance.

Several approaches have been used to differentiate disease resistance genes. These include the use of differential isolates of the pathogen, the test of allelism, the localization of the resistance genes in the host genome and the use of molecular markers.

Several classifications have been suggested for A. rabiei isolates based on the reactions of a set of differential host plants (Udupa et al. 1998; Chen et al. 2004; Chongo et al. 2004). However, the lack of universal differential host plants for isolate characterization and the use of different screening techniques or conditions, as well as the absence of a consensus as to whether the variability of A. rabiei is due to race or aggressiveness of a single race, make it difficult to distinguish different resistance genes using different pathogen isolates. Furthermore, reaction of different genotypes with potentially different genes for resistance to ascochyta blight often results in similar phenotypes. Therefore, resistance that may be contributed by different genes cannot be separated on the basis of disease evaluation alone. Molecular markers linked to the resistance genes offer an alternative tool for tracing genes for resistance to ascochyta blight. In addition, molecular markers can be used to assess the diversity at specific genomic regions that are associated with resistance to disease and to measure genetic relationships among genotypes. This approach has been used in wheat to separate germplasm with different resistance genes to fusarium head blight caused by Fusarium graminearum (McCartney et al. 2004).

To date, a number of Quantitative Trait Loci (QTL) for resistance to ascochyta blight were identified by different groups (Santra et al. 2000; Tekeoglu et al. 2002; Flandez-Galvez et al. 2003; Millan et al. 2003; Udupa and Baum 2003; Cho et al. 2004; Iruela et al. 2006). The use of common Simple Sequence Repeat (SSR) markers in most of these studies provided general conclusions that a major QTL on LG 2 close to the GA16 marker controlled the resistance to pathotype I of A. rabiei. Another region on LG2 at the proximity of TA37 locus also contributed to the resistance to pathotype I. Most reports demonstrated that the resistance to pathotype II is located on LG4. A number of SSR loci (GAA47, TA130, TR20, TA72, TS72 and TA2) were mapped within this region (Winter et al. 2000; Udupa and Baum 2003; Cho et al. 2004). By single-point analysis Cho et al. (2004) identified an additional SSR marker (TA46) that was strongly associated with the resistance derived from FLIP84-92C. This marker explained between 59% and 69% of the variation for resistance using different isolates under controlled environments; however, this marker did not show

linkage to other markers on the map. Using ICC12004 as the source of resistance, Flandez-Galvez et al. (2003) identified additional QTL for resistance to ascochyta blight under field conditions on a 5.6 cM interval between TS12b and STMS28 on LG1. Furthermore, a region flanked by TS45 and TA3b on LG2 was significantly associated with the disease reaction under controlled environments (Flandez-Galvez et al. 2003). The SSR marker loci on LG2 of the map reported by Flandez-Galvez et al. (2003) correspond to LG8 of the map constructed by Winter et al. (2000).

The current study used the available SSRs from previous mapping and QTL studies to evaluate the genetic relationships among 37 chickpea germplasm accessions differing in reaction to ascochyta blight. The chickpea germplasm accessions used for the analysis were derived from diverse geographical origins. The study provided information for the effective use of diverse genetic resources to improve ascochyta blight resistance in chickpea.

Materials and methods

Plant materials and field disease screening

One hundred and eighty-two chickpea germplasm accessions with putative resistance to ascochyta blight derived from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), International Centre for Agricultural Research in the Dry Areas (ICARDA), Washington State University, Regional Plant Introduction Station (WRPIS), United States Department of Agriculture (USDA) and Crop Development Centre, University of Saskatchewan collections were tested in the field in Saskatoon, Canada in the summers of 2003 and 2004. In this screening the germplasm accessions were exposed to naturally occurring mixed populations of *A. rabiei*. To increase the disease pressure, dried infected plant debris collected from chickpea production areas in Saskatchewan, Canada in the previous year was spread throughout the plot area and high humidity was maintained with misting irrigation. Thirty-five genotypes with the lowest disease scores (6 or lower using the same scale as for indoor disease screening) were selected and planted in the greenhouse for

further disease evaluation under controlled environments. The summary of indoor disease screening is presented in Table 1.

Indoor disease screening

A single-spore derived culture of *A. rabiei* isolate *ar68–2001* was used for indoor disease screening. The *ar68–2001* isolate was collected from cv. Sanford from a commercial production field in Saskatchewan in 2001. The isolate was selected for a high level of aggressiveness from a collection of more than 250 isolates obtained from different chickpea cultivars and production areas across Saskatchewan between 1998 and 2002. The isolate was grown at room temperature under continuous fluorescent light. Primary inoculum was produced by diluting 7 day-old colonies with sterile distilled water followed by agitating the cultures with a sterile glass rod. The suspensions were filtered through a Miracloth layer and adjusted to the final concentration of 2×10^5 conidia ml^{-1} using a hemacytometer. Tween 20 surfactant (polyoxyethylene sorbitan monolaurate) was added at a rate of one drop 100 ml^{-1} suspension.

Ten seeds of each chickpea genotype were grown in 10 cm square pots (1 seed per pot) in a greenhouse for four weeks. The plants were inoculated by spraying 2 ml of conidial suspension per plant or until run-off using an atomizer. Immediately after inoculation, the plants were transferred into a misting chamber covered with a translucent plastic sheet to provide 100% RH during the infection period. After 48 h incubation, the plants were transferred to a greenhouse bench. The temperature was maintained at 20/16°C (day/night) and 16 h photoperiod with fluorescent and incandescent lights. Plant reactions were scored visually two weeks after inoculation. Scoring was made on an individual plant basis on a scale of 0–9 (Singh and Reddy 1993; Chongo et al. 2004); where 0 = immune, no symptoms of disease; 1 = few, very small lesions (<2 mm^2) on leaves and stems (1–2% plant area infected); 2 = many, very small lesions and few small lesions (2–5 mm^2) on leaves and stems (3–5% plant area infected); 3 = many small lesions (6–10% plant area infected); 4 = few small and few large lesions (>5 mm^2), 11–25% plant area infected; 5 = many small and large lesions (26–50% plant area infected); 6 = many small and large lesions, lesions coalescing (51–75% plant area

Table 1 Seed type, status and origin of 37 chickpea germplasm accessions differing in reaction to ascochyta blight (AB)

Germplasm accessions	Seed type	Status	Origin/Breeding Institution	AB score ± Se
CDC Ebony	D	Cultivar	Univ. of Sask., Canada	4.8 ± 0.42
CDC Vanguard	D	Cultivar	Univ. of Sask., Canada	5.0 ± 0.45
304–31	D	Breeding line	Univ. of Sask., Canada	5.0 ± 0.66
304–40	D	Breeding line	Univ. of Sask., Canada	5.0 ± 0.52
95NN12	K	Breeding line	Univ. of Sask., Canada	4.6 ± 0.34
Amit	K	Cultivar	Bulgaria	4.5 ± 0.58
CDC Chico	K	Cultivar	Univ. of Sask., Canada	8.2 ± 0.52
CDC Frontier	K	Cultivar	Univ. of Sask., Canada	4.6 ± 0.52
FLIP82-150C	K	Breeding line	ICARDA, Syria	5.2 ± 0.62
FLIP83-48	K	Breeding line	ICARDA, Syria	5.5 ± 0.67
FLIP84-92C	K	Breeding line	ICARDA, Syria	4.8 ± 0.28
FLIP91-2	K	Breeding line	ICARDA, Syria	5.5 ± 0.64
FLIP91-46	K	Breeding line	ICARDA, Syria	5.6 ± 0.47
FLIP97-133C	K	Breeding line	ICARDA, Syria	5.2 ± 0.42
FLIP98-133C	K	Breeding line	ICARDA, Syria	5.3 ± 0.54
ICC76	D	Germplasm	India	4.9 ± 0.44
ICC1400	D	Germplasm	Unknown	5.1 ± 0.32
ICC1468	D	Germplasm	India	4.6 ± 0.54
ICC1532	D	Germplasm	Unknown	5.2 ± 0.64
ICC3996	D	Germplasm	India	4.0 ± 0.68
ICC4475	D	Germplasm	Unknown	4.5 ± 0.64
ICC4936	D	Germplasm	Greece	4.6 ± 0.48
ICC5124	K	Germplasm	India	5.3 ± 0.44
ICC12004	D	Germplasm	Unknown	4.0 ± 0.52
ICC12512-1	D	Germplasm	India	4.4 ± 0.37
ICC12952	D	Germplasm	India	5.0 ± 0.67
ICC12961	K	Germplasm	Former USSR	5.2 ± 0.52
ICC14911	K	Germplasm	Unknown	5.6 ± 0.44
ILC72	K	Germplasm	Former USSR	5.2 ± 0.34
ILC202	K	Germplasm	Former USSR	4.8 ± 0.72
ILC2506	K	Germplasm	Russia	5.2 ± 0.44
ILC2956	K	Germplasm	Former USSR	5.2 ± 0.47
ILC3279	K	Germplasm	Former USSR	4.8 ± 0.56
ILC3856	K	Germplasm	Morocco	4.8 ± 0.68
ILC5913	K	Germplasm	Unknown	5.6 ± 0.54
ILC5928	K	Germplasm	Morocco	5.0 ± 0.47
Sanford	K	Cultivar	USDA/ARS, USA	8.5 ± 0.50

Disease score was average of 10 plants under greenhouse conditions (Se = standard error)

infected); 7 = many small and large lesions, lesions coalescing, stem girdled (76–90% plant area infected); 8 = many small and large lesions, lesions coalescing, girdling stem breakage (>90% plant area infected), and 9 = plants dead. The disease score for each genotype was averaged from ten plants.

SSR analysis

Ten seeds of each genotype were grown in 10 cm square pots, one seed per pot, in a greenhouse. Two weeks after sowing, equal quantities of fresh leaf tissue from an average of eight plants of each

genotype were harvested and bulked for DNA extraction. Genomic DNA was prepared according to the protocol described by Doyle and Doyle (1990). The DNA was initially analyzed using 17 SSRs that were associated with the QTL for ascochyta blight resistance (Flandez-Galvez et al. 2003; Udupa and Baum 2003; Cho et al. 2004). Subsequently the DNA was analyzed using 24 SSRs from eight linkage groups of the chickpea SSR map (Tar'an et al. 2007) as a whole genome diversity analysis. Three SSRs that were distantly located from each other in each linkage group were selected. SSR loci that were linked to the QTL for ascochyta blight resistance were excluded from selection.

The SSR analysis was done following the protocol described by Winter et al. (1999). Both 10 bp and 50 bp DNA ladders were used as molecular weight markers for each gel. The SSR bands were visualized using silver staining protocol. The glass plates were scanned to create electronic files for band sizing and documentation. SSR allele sizing was done using AlphaEase software (Alpha Innotech Corporation, California, USA).

Polymorphic information content (PIC), genetic similarity and cluster analyses

PIC values were calculated with the following formula (Botstein et al. 1980):

$$PIC_i = 1 - \sum_{j=1}^{n} p^2_{ij}$$

Where n is the number of marker alleles for marker i, and p_{ij} is the frequency of the jth allele for marker i.

Band profiles were compiled onto a data matrix on the basis of the presence (1) or absence (0) of the allele bands. Genetic similarity (GS) between a pair of lines was calculated using the Dice index of similarity (Nei and Li 1979). Cluster analysis was conducted on the GS matrix using the UPGMA procedure of the NTSYS-pc programme version 2.02 g (Rohlf 1998). The resulting clusters were expressed as a dendrogram. The dendrogram presents a pictorial representation of the clustering process by indicating the order of individuals and groups joined together because of their similarity. The goodness of fit of the dendrogram was examined using Mantel's test for matrix correlation between the dendrogram and the GS (Mantel 1967). Two dendrograms, one based on the SSRs at the QTL regions and the other based on the SSRs from other regions of the chickpea genome, were constructed. The correspondence between the two dendrograms was tested with the Mantel Z statistic (Mantel 1967). This procedure examines the matrix-correspondence by taking the two matrices together and plots one against the other, element by element, except for the diagonal elements. This test gives the product-moment correlation, r, and a statistic test, Z, to measure the degree of relationship between two matrices. Significance of Z was determined by comparing the observed Z values with a critical Z value obtained by calculating Z for one matrix with 1,000 permuted variants of the second matrix. All computations were done with the NTSYS-pc programme version 2.02 g (Rohlf 1998).

Test populations

Three populations were developed by crossing moderately resistance lines with distinct SSR alleles at the QTL for the resistance to ascochyta blight. These crosses included ICC12004 × FLIP84-92C, ICC4475 × CDC Frontier and ICC3996 × Amit. A single F_1 plant from each cross was vegetatively propagated by stem cutting to maximize production of F_2 seeds for population development. Stimroot no.1 (Evergro Canada Inc., Delta, British Columbia, Canada) containing the active ingredient indole-3-butyric acid (IBA) was used to induce root development. Each F_2 plant was also vegetatively propagated. Three to four cuttings were made from each F_2 plant. Initially, the cuttings were grown in a peat pellet and incubated in a high humidity chamber with fluorescent light for about 10 days. The cutting-derived plants were then transferred into individual 10 cm square pots filled with Sunshine mix no. 4 medium (Sun Gro Horticulture Canada Ltd., Seba Beach, Alberta, Canada). Three cutting-derived plants from each F_2 plant that were relatively uniform in size from each population were selected. These cutting-derived plants served as replication in a completely randomized design in a greenhouse for disease evaluation using the same conditions and procedure as for indoor disease screening.

Analysis of variance (ANOVA) was done using the SAS package (SAS Institute Inc., 1999). Genotypic variance ($\sigma^2 g$) and phenotypic variance

($\sigma^2 p = \sigma^2 g + \sigma^2 e$) were determined based on expected mean squares of the ANOVA. Genetic coefficient of variation (GCV%) was calculated as % of the square root of genetic variance to population mean. The ratio of genetic variance to the total phenotypic variance served as the heritability estimate. Genetic advance (GA) was calculated based on the formula of $GA = k \times h^2 \times \sigma p$ (Falconer 1989), where k is a selection differential for which a standardized value (2.06) for 5% selection intensity was used in this analysis and σp is the standard deviation of the phenotypic variance. The GA is expressed as % of the mean population.

Results

There were only 35 germplasm lines and cultivars out of 182 accessions that consistently showed moderate to good resistance to ascochyta blight from the 2003 and 2004 field and greenhouse disease evaluations. These lines were selected for further disease evaluation and molecular characterization. Table 1 presents the mean reaction of the 35 chickpea germplasm accessions plus two susceptible cultivars (CDC Chico and Sanford) to *A. rabiei* infection under controlled conditions. Twenty lines had disease scores of 5.0 or lower. These lines had many small and few large lesions with less than 50% plant area infected. Six of these lines were selections made at the University of Saskatchewan, whereas the remaining genotypes in this category were germplasm lines originating from India, Greece, Russia, Morocco and ICARDA. Fifteen lines had disease scores ranging from 5.1 to 5.6. The disease scores for CDC Chico and Sanford (susceptible checks) were 8.2 and 8.5, respectively.

The 17 SSRs associated with the QTL for resistance to ascochyta blight used in the analysis detected 2 to 13 alleles (mean = 7.6) across Chico and Sandford and the 35 accessions and PIC values ranged from 0.47 to 0.87 with mean value of 0.71 (Table 2). On average the SSR loci on LG4 contained fewer alleles compared to the loci on LG2B (Table 2). The average PIC value of the SSR markers on LG4 (0.64) was much smaller than the average PIC value (0.82) of the markers in LG2A and LG2B combined. FLIP 84-92C and ILC3279 had identical SSR alleles for the QTL located on LG4 and LG8, except for the

Table 2 Summary of 17 SSR loci associated with QTL for resistance to AB in different linkage groups of the chickpea genetic map

Linkage group (Interval length)	SSR Locus	Number of alleles	PIC	Amplicon size range (bp)
LG1[a]	STMS28	6	0.54	230–252
	TS12	13	0.64	245–300
LG2A[b]	GA20	7	0.83	130–205
	GA16	8	0.84	230–275
LG2B[b]	TA37	5	0.69	258–300
	TR19	11	0.87	206–274
	TA22s	11	0.86	192–280
	TA176s	12	0.82	210–280
LG4[c]	GAA47	2	0.47	154–170
	TA130	7	0.60	180–230
	TR20	7	0.62	148–178
	TA72	8	0.68	220–256
	TA2	6	0.79	130–182
	TS72	7	0.69	230–295
LG8[c]	TS45	7	0.68	224–250
	TA3	4	0.69	260–294
Unassigned	TA46	6	0.51	150–178

Linkage assignment is based on [a] Flandez-Galvez et al. (2003); [b] Cho et al. (2004); [c] Winter et al. (2000)

TA72 locus; however, their alleles were distinctly different for the QTL on LG1 and LG2A + B (Table 3). ILC72, ICC12961 and ILC2956 had the same alleles for 16 SSR loci at all QTL regions. Available passport data and current analysis revealed that ILC72 and ICC12961 were derived from the same landrace. ILC 72 is maintained by ICARDA, while ICC12961 is maintained by ICRISAT. ICC3996 and ICC12004 also had identical SSR alleles on 15 loci (Table 3).

In LG4 the ILC72 alleles were identical with eight other genotypes (Table 3). In LG4, ILC3279 and ILC3856 had common alleles to that of ILC72, except for TA72 locus. Similarly for LG4, CDC Chico and Sanford had the same alleles as ILC72, except for the TA2 locus. Different alleles than that of ILC72 at all six loci on LG4 were found in a number of germplasms such as CDC Ebony, FLIP82-150C, ICC12952 and ICC3996.

Four genotypes, ILC72, ICC12961, ILC2956 and CDC Chico, had identical alleles at all four loci on

Table 3 SSR allele distribution across 37 chickpea germplasm accessions differing in reaction to ascochyta blight

Germplasm accessionss	LG1		LG2A		LG2B				LG4						LG8		Un
	STMS28	TS12	GA20	GA16	TA37	TR19	TA22s	TA176s	GAA47	TA130	TA72	TR20	TS72	TA2	TS45	TA3	TA46
CDCEbony	b	cl	a	a	b	e	f	i	b	f	d	f	c	d	e	ad	d
CDCVanguard	a	hj	b	b	b	e	h	be	b	c	a	c	e	f	g	bc	d
304–31	a	hj	b	b	b	e	h	be	b	c	a	c	e	f	f	b	d
304–40	a	hj	b	be	b	e	h	be	ab	c	a	c	e	f	f	b	d
95NN12	c	ci	d	h	e	h	e	i	b	e	g	b	a	e	e	a	c
Amit	c	hj	c	a	a	h	b	j	a	c	a	c	f	b	c	a	d
CDCChico	ab	eg	e	f	a	d	e	h	a	c	a	c	e	a	ab	a	d
CDCFrontier	c	hj	f	g	a	g	g	i	a	c	a	c	e	b	c	b	d
FLIP82-150C	c	c	d	e	e	f	d	i	b	e	g	b	e	e	d	b	c
FLIP83-48	c	e	f	g	a	d	e	g	a	c	a	c	e	b	c	c	d
FLIP84-92C	c	hj	f	g	b	g	g	i	a	c	a	c	e	b	c	b	d
FLIP91-2	c	e	f	g	d	g	g	f	a	c	a	c	e	f	c	c	d
FLIP91-46	a	c	f	g	b	g	k	j	a	c	a	c	e	b	c	c	d
FLIP97-133C	c	hj	f	g	b	g	g	h	a	c	a	c	e	b	c	b	c
FLIP98-133C	b	eh	c	d	a	f	i	h	a	b	b	f	c	e	bd	c	d
ICC12004	e	bd	g	h	d	a	b	d	b	f	d	f	b	d	e	d	d
ICC12512-1	f	fl	c	a	a	e	f	i	b	c	a	d	d	c	f	a	a
ICC12952	d	g	e	f	b	f	f	i	b	b	b	d	d	c	d	a	b
ICC12961	c	hj	e	f	a	d	e	h	a	c	a	c	e	b	c	a	d
ICC1400	b	fj	d	e	c	e	h	k	b	b	a	c	b	c	c	c	a
ICC1468	o	cd	g	h	a	c	f	a	b	f	h	d	b	d	c	d	d
ICC14911	b	c	d	f	a	e	i	j	b	e	c	e	g	a	e	c	b
ICC1532	b	lm	e	e	b	j	c	f	b	a	e	g	f	a	e	b	e
ICC3996	e	bc	g	h	d	a	a	d	b	f	f	f	b	c	e	d	e
ICC4475	e	ad	g	h	d	b	a	c	b	f	f	f	b	c	d	d	d
ICC4936	d	g	d	f	a	f	f	h	b	b	b	d	d	c	d	a	b
ICC5124	c	hj	b	c	a	f	j	l	a	d	b	a	e	d	c	b	f
ICC76	e	bd	g	h	d	f	b	b	b	f	f	f	d	c	e	d	d
ILC202	c	c	e	f	c	i	e	h	a	c	a	c	d	c	e	c	d
ILC2506	c	hj	c	a	b	h	b	i	a	c	a	c	e	b	c	a	d
ILC2956	c	hj	e	f	a	d	e	h	a	c	a	c	e	b	c	a	d

Table 3 continued

Germplasm accessionss	LG1		LG2A		LG2B				LG4						LG8		Un
	STMS28	TS12	GA20	GA16	TA37	TR19	TA22s	TA176s	GAA47	TA130	TA72	TR20	TS72	TA2	TS45	TA3	TA46
ILC3279	c	c	e	f	b	i	e	h	a	c	b	c	e	b	c	b	d
ILC3856	c	hj	c	a	b	h	b	i	a	c	b	c	e	b	c	a	d
ILC5913	a	f	b	b	b	c	d	l	b	c	a	c	e	a	c	b	c
ILC5928	c	hk	d	a	b	i	e	i	a	c	b	c	F	a	c	a	c
ILC72	c	hj	e	f	a	d	e	h	a	c	a	c	E	b	c	b	d
Sanford	b	eg	e	f	a	d	e	i	a	c	a	c	E	a	b	b	d

Accessions with the same letter at a given SSR locus share the same allele

LG2B. Two additional genotypes (Sanford and FLIP83-48) also had alleles that were common with ILC72, except the allele for the TA176s locus (Table 3). Three desi genotypes (CDC Vanguard, 304–31 and 304–40) which are sister lines, had common alleles on LG2B region. There were ten and seven genotypes that had identical SSR alleles with ILC72 on LG1 and LG2A, respectively. At LG8, seven genotypes had alleles in common with ILC72. These included two resistance sources ILC3279 and FLIP84-92C.

The result of UPGMA cluster analysis based on the 17 SSRs associated with the QTL for resistance to ascochyta blight is presented in Fig. 1. The cluster analysis grouped the chickpea genotypes with identical SSR alleles and tended to group the genotypes based on seed types and country of origin. The Mantel Z test statistics showed a non-significant correlation ($r = 0.16$; $P = 0.99$) between the matrix of genetic relationships based on the QTL regions (Fig. 1) and the matrix of genetic relationships based on the whole genome (Fig. 2).

For the dendrogram based on the QTL regions, three groups were distinguished by truncating the dendrogram at the GS value of 0.164 (Fig. 1). The first group consisted of five germplasm accessions and cv. CDC Ebony, which was derived from a cross between ICC7524 and ICC1468. The germplasm in this group included ICC76, ICC1468, ICC3996, ICC4475 and ICC12004, which all are desi type. Each genotype in this group had a disease score of 5.0 or lower. Two were from India, while the origin of the others is unknown.

Seven genotypes, 4 desi and 3 kabuli, were clustered in group II. Two of the kabuli types in this group, 95NN12 and FLIP82-150C, are breeding lines developed at the University of Saskatchewan and ICARDA, respectively, whereas the origin of ICC14911 is unknown. Of the four desi types, two were collected from India, one from Greece and one of unknown origin.

Group III formed the major cluster, which consisted of twenty-three genotypes. The majority of genotypes in group III are of the kabuli seed type, except for CDC Vanguard, 304–31 and 304–40. Four subclusters were visible within group III at the cut-off value of 0.52. These subclusters tended to group the genotypes based on their country of origin, breeding institution or pedigree. CDC Vanguard and its sister lines (304–31 and 304–40) derived from a complex

Fig. 1 UPGMA cluster analysis of 37 chickpea germplasm accessions differing in reaction to ascochyta blight based on 17 SSR loci at the QTL regions. Groups of accessions based on the cut-off value of 0.164 are indicated on the right
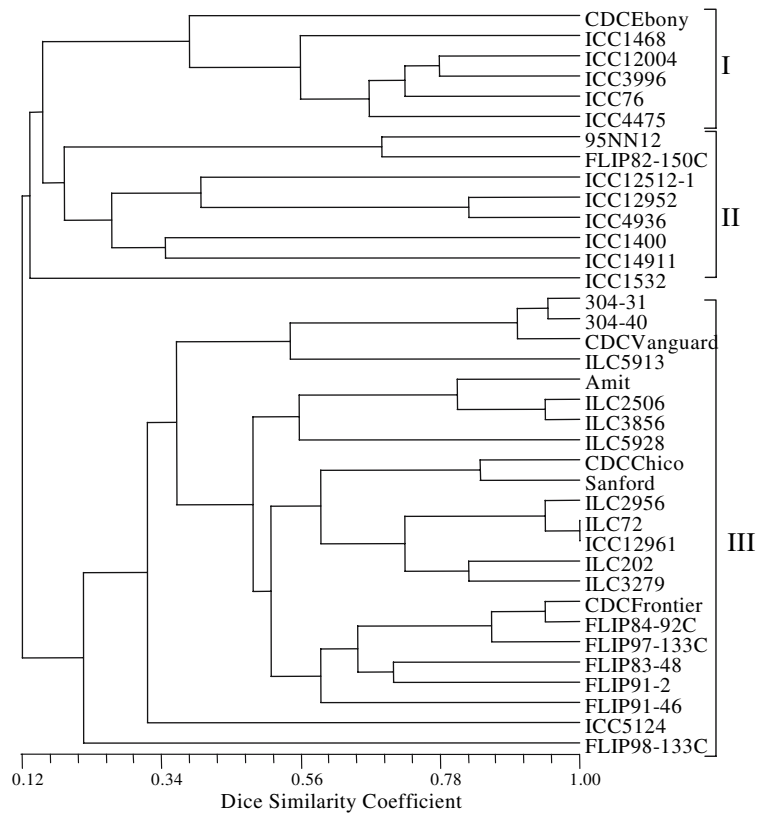

cross involving ICRISAT selections were grouped together with ILC5913. ILC2506, ILC3856 and ILC5928 were placed together with cv. Amit. Two genotypes (ILC3856 and ILC5928) in this subcluster originated from Morocco, whereas Amit was a selection from a landrace originating in Bulgaria. Five genotypes (ILC2956, ILC72, ICC12961, ILC202, ILC3279) which are landraces collected in the former Soviet Union formed a separate subcluster. Two susceptible cultivars (CDC Chico and Sanford) were placed within this group. The fourth subcluster consisted of five breeding lines developed at ICARDA and a cultivar (CDC Frontier) released by the University of Saskatchewan. Several genotypes that are distantly related based on the SSRs at the QTL regions were identified. These included ICC3996, ICC12004 and ILC2956.

Figure 3 shows the distribution of the disease scores in three segregating populations that were developed by crossing parental lines with distinct SSR alleles at the QTL for ascochyta blight resistance. For population A, the disease score of the F_2 plants ranged from 3.6 to 6.8 (mean = 4.35) while the disease score of ICC12004

and FLIP84-92C was 4.4 and 4.9, respectively. In population B, the scores of ICC4475 and CDC Frontier were 4.4 and 4.7, respectively. Disease scores varing from 3.5 to 7.0 were observed among the F_2 plants derived from the ICC4475 and CDC Frontier cross. The same trend was also observed on population C derived from the ICC3996 × Amit cross from which disease scores ranging from 3.4 to 7.0 were observed. F_2 plants both with enhanced levels of resistance and higher disease scores than the parents were found in each of the F_2 populations. Moderate amounts of genetic coefficient of variations (19–25%) exist within these populations (Table 4). Broad sense heritability estimates ranged from 0.38 to 0.43. At 5% selection intensity, the estimated genetic gain as % of the mean population ranged from 12% for ICC12004 × FLIP82-94C cross to 16% for ICC3996 × Amit cross suggesting that there were some different alleles for resistance to asscochyta blight, in the two parental lines. These results suggest that it is possible to develop chickpea cultivars with a higher level of resistance by accumulating resistance alleles from genetically distant sources.

Fig. 2 UPGMA cluster
analysis of 37 chickpea
germplasm accessions
differing in reaction to
ascochyta blight based on
24 SSR loci distributed over
eight linkage groups of the
chickpea linkage map
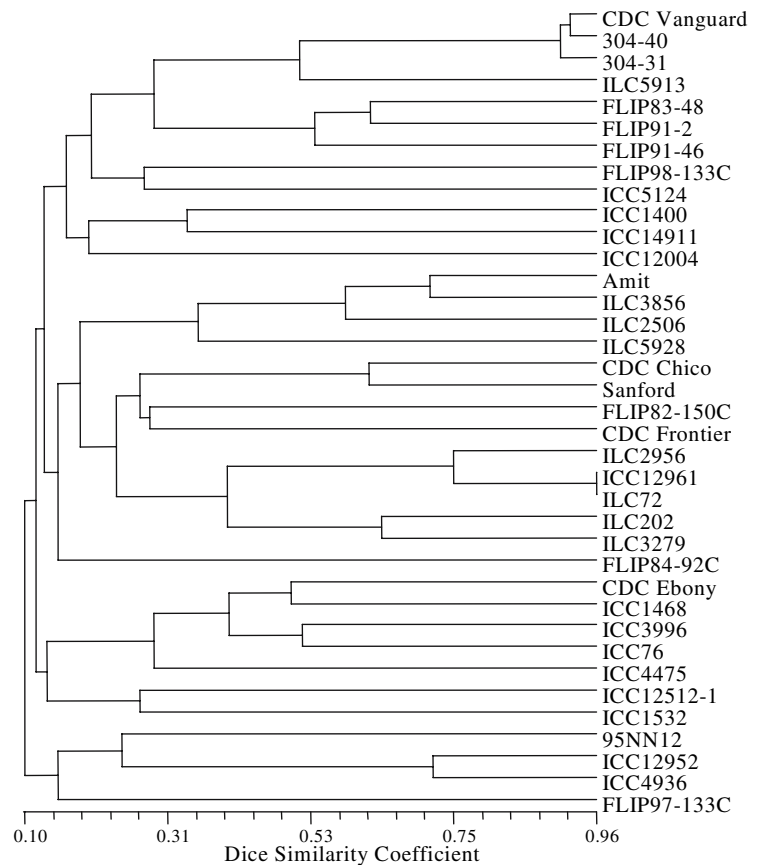
Discussion

The current study provides an illustration of allele
diversity at SSR loci associated with QTL for
ascochyta blight resistance across a diverse collection
of chickpea germplasm accessions. The hierarchical
clustering based on these SSR alleles enabled us to
differentiate three major groups of these chickpea
germplasm accessions differing in reaction to asco-
chyta blight (Fig 1). The largest group (group III) was
dominated by accessions of kabuli seed type. Within
this group, the SSR alleles of the landraces collected
from the former Soviet Union such as ILC72,
ILC2506, ILC2956 and ILC3279 were the most
common among the kabuli genotypes. This was
expected since ILC72 and ILC3279 have been widely
used as sources of ascochyta blight resistance around
the world (Crino 1990; Muehlbauer et al. 1998, 2004;
Muehlbauer and Kaiser 2002; Millan et al. 2003;
Rubio et al. 2004). Pedigree information also dem-
onstrated that ILC72 was used as the donor for the

resistance in FLIP84-92C (Tekeoglu et al. 2000).
FLIP84-92C is a moderately resistant germplasm
accession that has been frequently used for studying
the genetics of resistance to ascochyta blight (Santra
et al. 2000; Tekeoglu et al. 2002; Cho et al. 2004).
Our disease screening revealed that FLIP84-92C had
slightly better resistance to ascochyta blight com-
pared to ILC72 suggesting that FLIP84-92C may also
have inherited the resistance alleles from the other
parent (ILC215). Two cultivars (CDC Chico and
Sanford), which were initially released as moderately
resistant to ascochyta blight, also had ILC72 in their
background. Under Saskatchewan conditions, the
occurrence of new pathotypes or the increased of
aggressiveness of the current of *A. rabiei* pathotypes
has overcome the resistance in these cultivars. Our
analysis demonstrated that CDC Chico and Sanford
shared common SSR alleles with ILC72 on LG2A,
LG2B and LG 4 except for TA176s and TA2 loci on
LG2B and LG4, respectively. Their SSR profiles were
distinctly different for the QTL regions at LG1 and
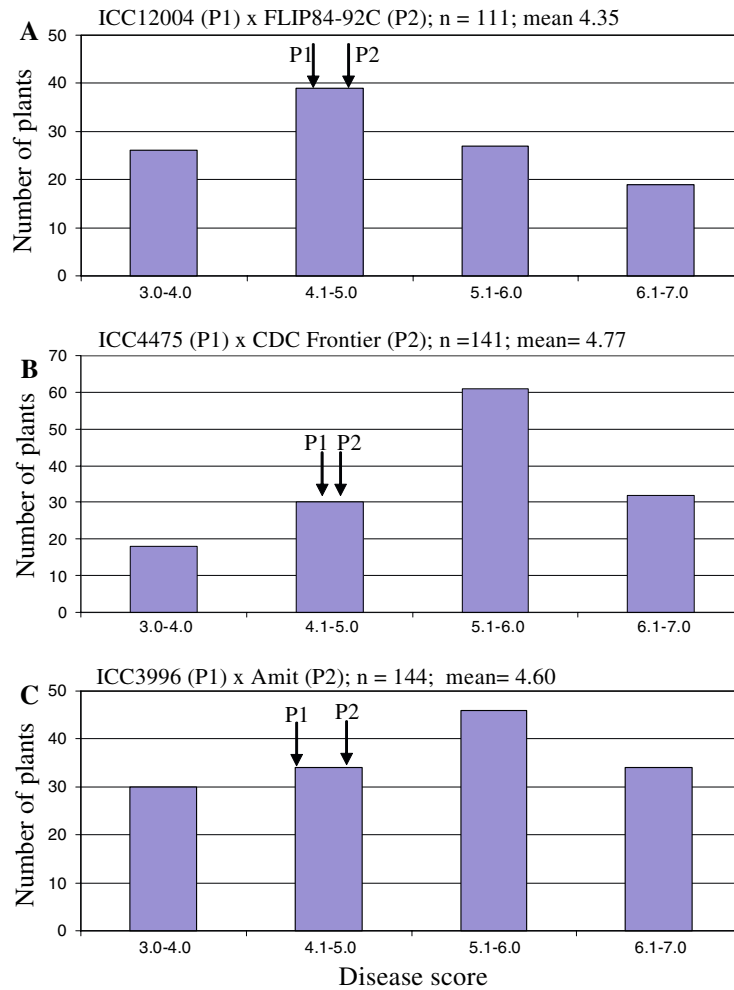
Fig. 3 Frequency distributions of three F_2 populations of chickpea derived from crosses of moderately resistant parents (A = ICC12004 × FLIP84-92C; B = ICC4475 × CDC Frontier; C = ICC3996 × Amit). The number of F_2 plants (n) for each population and mean disease score for each populations are presented. Arrows show disease score for each parental line in each population. The disease was rated using a 0 to 9 scale, where 0 = no symptoms and 9 = plants dead

LG8. Further analyses are needed to examine if these differences may contribute to the maintenance of resistance in ILC72 under Saskatchewan conditions.

The SSR allele diversity analysis demonstrated a highly conserved allele combination for the SSR across the QTL regions on LG4 compared to the QTL on other linkage groups. These results suggested that this region might have been targeted for selection for ascochyta blight resistance reducing the overall variation compared to other genomic regions.

Table 4 Mean ascochyta blight (AB), genetic coefficient of variation (GCV), heritability (H^2) and predicted genetic advance (GA) of three F_2 populations derived from crosses of moderately resistant genotypes

Cross	Population size	Mean AB	GCV (%)	H^2	GA (%)
ICC12004 × FLIP84-92C	111	4.35	19	0.38	12
ICC4475 × CDC Frontier	141	4.77	23	0.41	14
ICC3996 × Amit	144	4.60	25	0.43	16

The relationships among the chickpea germplasm accessions as revealed by the SSR alleles at the QTL regions were not correlated with those based on the SSR loci derived from other regions of different linkage groups, suggesting that the diversity at the QTL regions may not reflect the overall diversity at the whole genome. However, to some extent, sub-clusters containing few genotypes that had common parents in their pedigree were consistent on both dendrograms. For example, the sub-cluster of CDC Vanguard, 304–431 and 304–40, which are sister lines, were clustered together on both dendrograms.

Several genotypes such as ICC3996, ICC12004 and ILC2956 were distantly related based on the SSRs at the QTL regions. These lines might be used as sources of resistance to broaden the genetic base for the newer cultivars. For example, the SSR alleles on LG2A + B and LG4 in ICC3996 were relatively rare in this germplasm collection and were completely different from those in ILC72 and ILC3279. Moderate amounts of genetic variability exist within the population derived from a cross between ICC3996 and Amit. Disease evaluation of this population demonstrated some transgressive segregants with enhanced resistance and some with increased susceptibility compared to the parents. These results suggested that there were different minor resistance genes with additive gene actions in each of the two parental lines. The same results were also found from the other test populations from crosses between ICC12004 × FLIP84-92C and ICC4475 × CDC Frontier. The estimated genetic gain from selection in these three test populations averaged 14%. These results suggest that it is possible to develop chickpea cultivars with a higher level of resistance by accumulating resistance genes from different sources.

The current analysis of the genetic diversity using SSRs at the QTL regions associated with resistance to ascochyta blight suggest that the SSR alleles of the germplasm originating from the former Soviet Union were relatively common among the collection of chickpea germplasm accessions used in the study. Available pedigree information also showed that only a few sources were widely used in breeding pro-grammes to develop resistant cultivars. Several potential sources of resistance from germplasm or breeding lines from different geographical origins may be used in breeding programmes in combination with adapted varieties to develop better and possibly more durable resistance to ascochyta blight. The current analyses provided information on genotypes with distinct genetic backgrounds at genomic regions associated with the QTL for ascochyta blight resis-tance. Our evaluation using three F_2 populations derived from crosses of moderately resistance parents with diverse genetic backgrounds at regions associ-ated with resistance suggest that it is possible to recover progeny with better resistance to ascochyta blight than either parent.

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FULL RESEARCH PAPER

Inheritance of resistance to *Mycosphaerella pinodes* in two wild accessions of *Pisum*

Sara Fondevilla · José I. Cubero · Diego Rubiales

Abstract *Mycosphaerella pinodes* is one of the most devastating pea pathogens. Pea cultivars with adequate levels of resistance to control the disease are not so far available. However, promising levels of resistance have been identified in wild accessions of pea. In the present investigation the inheritance of resistance to *M. pinodes* was studied in two crosses between the susceptible pea cv. 'Ballet' and the partially wild resistant accessions P665 (*Pisum sativum* subsp. *syriacum*) and P42 (*P. sativum* subsp. *sativum* var. *arvense*). Both additive and dominant effects were important in control of resistance and susceptibility dominated over resistance.

Keywords Ascochyta blight · Pea · Genetic

Introduction

Pea is the most commonly produced grain legume in Europe and second-most in the world (FAOSTAT data, 2005; http://faostat.fao.org/). Ascochyta blight, caused by *Mycosphaerella pinodes*, the teleomorph of *Ascochyta pinodes*, is one of the most important pea pathogens (Moussart et al. 1998). It is widespread throughout the major pea-growing areas, especially in temperate regions of Europe, North America, Australia and New Zealand (Wallen 1965; Lawyer 1984; Bretag et al. 1995). Average yield losses in commercial pea fields have been estimated at 10%, and losses of >50% have been measured in some trials (Xue et al. 1997). The disease reduces number of seeds per stem and seed size (Tivoli et al. 1996).

Management of the disease by fungicide seed treatment, crop rotation and sanitation is possible, but each has deficiencies. Resistance appears to be the more practical way to reduce its effects (Zimmer and Sabourin 1986). Although extensive screening of pea germplasm has been conducted, only partial resistance has been identified that by itself, is inadequate to control the disease. Good levels of partial resistance have been reported in wild pea accessions (Zimmer and Sabourin 1986; Clulow et al. 1991a, Wroth 1998; Fondevilla et al. 2005). Knowledge of the genetic factors controlling resistance to *M. pinodes* in these wild accessions would facilitate gene transfer to pea cultivars. With this aim, the present work examines the inheritance of resistance to *M. pinodes* in two partially resistant wild accessions of pea.

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Materials and methods

Plant material

Two partially resistant accessions P42 (*Pisum sativum* subsp. *sativum* var. *arvense*) and P665 (*P. sativum* subsp. *syriacum*) (Fondevilla et al. 2005) were crossed with the susceptible commercial cultivar 'Ballet' (*P. sativum* subsp. *sativum*). The derived F_1 plants of both crosses were evaluated for resistance to *M. pinodes* and selfed to obtain the F_2 generation. In addition, the reaction to *M. pinodes* was examined in backcrosses obtained by crossing F_1 plants derived from the cross P665 × 'Ballet', with 'Ballet' (BC_1) and P665 (BC_2). The testa of seeds was pierced to aid inhibition before seeds were germinated and sown, one seed per pot, containing 440 cm^3 of 1:1 sand–peat mixture. Plants were grown in a glasshouse to the 3–4 leaf stage (approximately 14 days after planting). They were then transferred to a growth chamber (21 ± 2°C with a 12 h dark/12 h light photoperiod, at 106 µmol m^{-2} s^{-1}) and arranged according to a complete randomised design for inoculation.

Inoculation and incubation

Plants were inoculated using the monoconidial isolate CO-99 obtained from infected pea plants collected in commercial fields at Córdoba (Spain). The isolate was multiplied in Petri dishes of V8 medium (200 ml of V8 vegetable juice + 40 g of technical agar + 800 ml of sterile water litre^{-1}) at 23°C, subjected to a 16 h photoperiod of fluorescent illumination at 27 µmol m^{-2} s^{-1}. A spore suspension was prepared by flooding the surface of 12–14 day-old cultures with sterile water, scraping the colony with a needle and filtering the suspension through two layers of sterile cheesecloth. The concentration of spores was determined with a haemocytometer and adjusted to $5 × 10^5$ spores ml^{-1}. Tween-20 (120 µl 100 ml^{-1} of spore suspension) was added as a wetting agent and the spore suspension was applied with a sprayer at a rate of 1 ml per plant. After inoculation, plants were incubated in a growth chamber at 21 ± 2°C with a 12 h dark/12 h light photoperiod, the first dark period commencing immediately after inoculation. During the first 24 h, plants were covered with a polyethylene sheet and high humidity was ensured by ultrasonic humidifiers operating for 15 min every 2 h. The polyethylene cover was then removed.

Disease assessment

Disease was visually assessed 14 days after inoculation using a 0–5 scale defined by Roger and Tivoli (1996) as follows:

0 = no lesions
1 = a few scattered flecks
2 = numerous flecks
3 = 10–15% of the leaf area necrotic and appearance of coalescent necrosis
4 = 50% of the leaf area dehydrated or necrotic
5 = 75–100% of the leaf area dehydrated or necrotic.

Average disease rating (DR) for each plant was defined as the mean disease score over its first, second and third leaves.

Data analysis

The joint scaling test proposed by Cavalli (Mather and Jinks 1971, pp 71–76) was used to analyse data. The test checks the conformity with the additive-dominance model and gives additional information about the weight of dominance and additive effects in the control of the trait. Cavalli's test estimates the parameters 'm', 'd' and 'h', from means of the available types of generations; with 'm' defined as the mid-parental value, 'd' as the half of the difference between parental values and 'h' as the deviation of F_1 generation from their respective mid-parental values. Subsequently, the observed generation means were compared with expected values derived from the estimates of the three parameters assuming that the cross fitted the additive-dominance model. In this study this comparison was performed by using the chi-square (χ^2) test and linear regression.

The requirements of the additive-dominance model are (I) normal diploid segregation of chromosomes, (II) homozygous parents, (III) no genotype by environment interaction, (IV) no reciprocal differences, (V) no epistasis, (VI) no uncorrelated gene distribution and (VII) no multiple alleles (Hill 1964).

Broad sense heritabilities (H) were calculated by dividing the genetic component (additive + domi-

nance) by the total variance (σ^2) (additive + dominance + environmental components) as follows:

$$H = (\sigma^2_{F2} - \sigma^2_M) / \sigma^2_{F2}$$

The environmental components (σ^2_M) was estimated using the formula:

$$\sigma^2_M = 1/3 \ (\sigma^2_{F1} + \sigma^2_{P1} + \sigma^2_{P2})$$

Results and discussion

Accessions P665 and P42 were partially resistant to *M. pinodes*, confirming previous reports (Fondevilla et al. 2005). Thus, 2 weeks after inoculation P665 and P42 showed DRs of 2.5 and 3.2, respectively, while the DR for the highly susceptible 'Ballet' was 4.77 (Tables 1 and 2). The F_1 derived from both crosses were as susceptible as 'Ballet'. That was also the case of the BC_1 obtained from the cross 'Ballet' × P665. In contrast, the BC_2 generation of this cross displayed a DR higher than P665 but lower than 'Ballet'. In the F_2 of both crosses the DR showed a continuous distribution skewed towards susceptibility (Fig. 1). The cross P665 × 'Ballet' fitted the additive-dominance model (Table 1; χ^2 $P > 0.05$; linear regression $P < 0.05$). In this cross, parameters 'd' and 'h' were

Table 1 Summary of conformity of the 'Ballet' × P665 cross to the additive-dominance model

Generation	Number of plants	Observed values[a]	Expected values[b]
'Ballet'	17	4.77	4.79
P665	6	2.50	2.63
F_1	5	4.45	4.83
F_2	153	4.34	4.27
BC_1	7	4.81	4.81
BC_2	3	3.40	3.73
$\chi^2_{(gl = 3)}$		3.71 not significant	
R^{2c}		0.959	

[a] Disease rating visually assessed using a 0–5 scale defined by Roger and Tivoli (1996)

[b] Expected values derived from joint scaling test proposed by Cavalli (Mather and Jinks 1971)

[c] R^2 and significance of linear regression

*** Significance levels $P < 0.001$

Table 2 Summary of conformity of the P42 × 'Ballet' cross to the additive-dominance model

Generation	Number of plants	Observed values[a]	Expected values[b]
P42	20	3.2	3.46
'Ballet'	17	4.77	4.80
F_1	4	4.83	5.11
F_2	167	4.67	4.62
$\chi^2_{(gl = 1)}$[b]		5.16*	
R^{2c}		0.958*	

[a] Disease rating visually assessed using a 0–5 scale defined by Roger and Tivoli (1996)

[b] Expected values derived from joint scaling test proposed by Cavalli (Mather and Jinks 1971)

[c] R^2 of linear regression

* Significance level $P < 0.05$

significantly different from zero showing that both additive and dominance effects were involved in the control of the resistance (Table 3). In addition, 'h' and 'd' gave similar positive values suggesting a complete dominance of susceptibility over resistance. Broad sense heritability displayed a value of 0.43 (Table 5).

Concerning the cross P42 × 'Ballet', although according to the χ^2 value the additive-dominance model should be rejected for this cross, the linear regression showed that the observed values for each generation were highly correlated with the expected values (Table 2). As the F_2 generation values depends on the parental and F_1 values, the χ^2 value could be inflated and, therefore, we cannot rule out the possibility that the cross P42 × 'Ballet' fits the additive-dominance model. If that were the case, the estimation of the additive and dominant effects by Cavalli's test would be accurate and we could conclude that in cross P42 × 'Ballet', as in 'Ballet' × P665, both dominant and additive effects contribute in the control of the resistance (Table 4). In this cross, broad sense heritability was higher than in the cross 'Ballet' × P665 and showed a value of 0.60 (Table 5).

In the two crosses analysed, the distribution of DR was normal suggesting that resistance is a polygenic trait. Other possibilities might be that resistance is controlled by a single or a few major genes whose expression is highly influenced by the environment. Whatever the case, as F_2 individuals could not be

Fig. 1 Histograms of disease rating (Roger and Tivoli 1996) measured in the F$_2$ derived from the crosses 'Ballet' × P665 (**a**) and P42 × 'Ballet' (**b**). Arrows indicate parental, F$_1$, BC$_1$ and BC$_2$ values
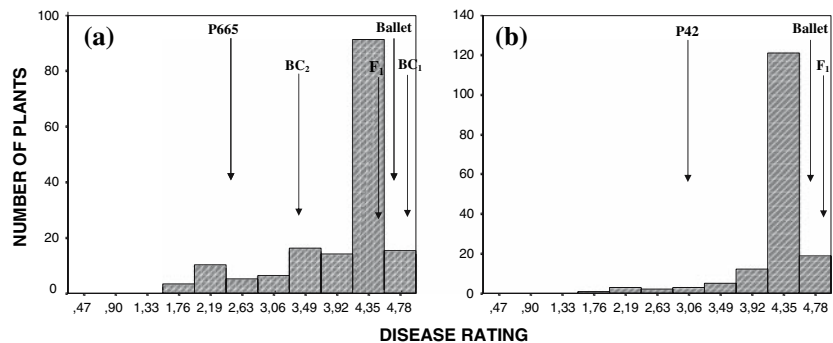
Table 3 Summary of the Cavalli's test for the 'Ballet' × P665 cross

Parameter	Value	S[a]	t-student
m[b]	3.712	0.106	34.866***
d[c]	1.077	0.108	9.998***
h[d]	1.122	0.218	5.152***

[a] Standard deviation of the parameter

[b] Mid-parent value

[c] Half of the difference between parental values

[d] Deviation of F$_1$ from their respective parent values

*** Significance level $P < 0.001$

Table 4 Summary of the Cavalli's test for the P42 × 'Ballet' cross

Parameter	Value	S[a]	t-student
m[b]	4.128	0.103	40.162***
d[c]	0.672	0.110	6.125***
h[d]	0.987	0.199	4.962***

[a] Standard deviation of the parameter

[b] Mid-parent value

[c] Half of the difference between parental values

[d] Deviation of F$_1$ from their respective parent values

*** Significance level $P < 0.001$

Table 5 Estimates of broad sense heritability in two crosses between the pea variety 'Ballet', susceptible to *M. pinodes*, and the partially resistant wild pea accessions P665 and P42

	'Ballet' × P665	P42 × 'Ballet'
Genetic variance (σ_{F2}^2)	0.83	0.41
Environment variance (σ_M^2)	0.47	0.16
Broad sense heritability (H)	0.43	0.60

ambiguously classified in resistance classes, the trait has to be treated as a quantitative character. Our results agree with the majority of previous studies reporting that the inheritance of resistance to *M. pinodes* in pea is controlled by a complex system. Thus, Wroth (1999), using biometric approaches, concluded that resistance to *M. pinodes* in pea was controlled by a polygenic system. Similarly, mapping the resistance to ascochyta blight in several pea crosses has resulted in the identification of numerous genomic regions controlling the trait (Timmerman-Vaughan et al. 2002, 2004; Prioul et al. 2004). In contrast, Clulow et al. (1991b) was able to separate individuals of segregrant populations into discrete resistance classes and concluded that in some crosses resistance was dominant and controlled by single genes.

In this study we report genetic analysis in wide crosses between different subspecies of *Pisum*, where distorted segregations could be expected. However, both crosses gave a good fit to the additive-dominance model showing that, at least for the character studied in this paper, genes are segregating in Mendelian ratios. In addition, as the absence of epistatic effects is an assumption of Cavalli analysis, the conformity of the crosses with the model implies that gene interactions do not play an important role in the control of the resistance. The absence of gene interactions and the presence of additive effects leads to the possibility of enhancing the level of resistance to *M. pinodes* by gene pyramiding.

The analysis performed revealed that the dominance component was also important in the control of resistance. F$_1$ individuals derived from both crosses were as susceptible as the susceptible parent 'Ballet' and the distribution of the DR in the F$_2$ were skewed towards susceptibility. These facts show that suscep-

tibility is dominant over resistance in the two crosses. A similar outcome of recessive genes controlling resistance to *M. pinodes* was reported by Ali (1983) while in other pea germplasm the resistance to this pathogen is of dominant nature (Wroth 1999; Clulow et al. 1991b). Although dominance effects will disappear in advanced breeding material, they have to be taken in account in the early stages of breeding programmes including accessions P665 and P42. Thus, the recessive nature of the resistance implies that selection must be performed in selfed generations.

Wroth (1999) found that the inheritance of resistance to *M. pinodes* in leaves fitted the additive-dominance model in some crosses between *P. sativum* accessions. In contrast, the model was rejected for disease response in stems. In two of these crosses, the genetic variance was mainly attributed to additive effects, whereas dominance effects were more important in a third cross.

The moderate value of broad sense heritability showed that resistance expression was influenced by the environment. Several investigations have pointed out the strong importance of environmental factors such as temperature and humidity in the development of ascochyta blight (Wroth 1999; Roger et al. 1999a, b). This result was confirmed in our study where genetic studies were performed under controlled environmental conditions, and differences in the level of resistance were identified within individuals of non-segregating generations. For instance, F_1 showed great variance, contributing to the high value of the environment component estimated. The high variance observed may be caused by the low number of F_1 individuals that were screened in both crosses. Consequently, it is possible that heritability was underestimated in our study. In fact, F_2 individuals at least as resistant as their respective resistant parents were observed in both crosses, suggesting that the heritability values allow for an appropriate strategy of selection for greater resistance.

As resistance is present in the non-adapted pea accessions and is quantitative and recessive, a recurrent selection scheme will be a suitable breeding strategy. In the proposed breeding programme, both wild lines will be crossed to commercial cultivars, both F_1s selfed and the F_2 screened under field conditions to select the best plants showing the highest possible degree of resistance as well as good

agronomic features. The best F_2 plants will be backcrossed with the commercial cultivar, starting a new crossing cycle. They will also be advanced to F_3 progenies, where disease and general assessment is more accurate. The lines selected will be intercrossed in as many combinations as possible. The descendants of these crosses will be further selfed, screened for resistance and agronomic characteristics, crossed again and with commercial cultivars. This method has proved to be efficient in several crops (for example, in soybean; Wilcox 1998) in accumulating polygenic alleles for resistance in a common genotype. The method for autogamous species is much more time-consuming than for outcrossers and would be specially tedious when wild accessions are involved. However, in the absence of good levels of resistance to *M. pinodes* in cultivated pea, the effort is worthy to enhance the level of resistance to this worldwide important pea disease.

The studies described here represent the first step towards the development of pea lines with increased resistance. Our conclusions are based on experiments performed under controlled conditions at the seedling stage and using only one isolate. Therefore, our results may differ from those obtained with naturally infected mature field plants. However, previous studies performed with several wild pea accessions showing different levels of resistance to *M. pinodes* have proved that disease assessments under controlled conditions provide a good estimation of field resistance (Fondevilla et al. 2005). Furthermore, accession P665 was found to be resistant against different *M. pinodes* isolates originating from different countries, showing that the resistance present in this accession is not isolate-specific.

The biometric approach performed in early generations was selected from other possible methods because it allows the estimation of the dominance component, providing early and useful information for planning breeding strategies. Future research will include the mapping of genomic regions involved in the control of resistance to *M. pinodes*. This approach would enhance our current knowledge about the genetics of the trait and may be useful to validate the conclusions derived from the present study. With this aim, a population of recombinant inbred lines derived from a cross between accession P665 and the susceptible variety 'Messire' is being developed.

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FULL RESEARCH PAPER

Comparison of the epidemiology of ascochyta blights on grain legumes

Bernard Tivoli · Sabine Banniza

Abstract Asochyta blights of grain legumes are caused by fungal pathogens in the genus *Ascochyta*. Different species infect the different legume species, and in pea three species including *Phoma medicaginis* var. *pinodella* have been implicated in ascochyta blight. The impact of the diseases varies between crops, countries, seasons and cropping systems, and yield loss data collected under well-defined conditions is scarce. However, ascochyta blights are considered major diseases in many areas where legumes are grown. Symptoms appear on all aerial parts of the plant, and lesions are similar for most of the species, except for *M. pinodes* and *P. medicaginis* var. *pinodella*. Infected seed, stubble and/or air-borne ascospores are major sources of primary inoculum. Their importance varies between species and also between regions. All *Ascochyta* spp. produce rain-splashed conidia during the cropping season which are responsible for the spread of the disease within the crop canopy. Only in pea are ascospores involved in secondary disease spread. Limited data suggests that *Ascochyta* spp. may be hemibiotrophs; however, toxins characteristic for necrotrophs have been isolated from some of the species. Modelling of ascochyta blights is still in the developmental stage and implementation of such models for disease forecasting is the exception.

Keywords Pea · Faba bean · Chickpea · Lentil · *Ascochyta* · *Mycosphaerella pinodes* · *Phoma medicaginis* · *Didymella* · Life-cycle

Introduction

Grain legumes, also referred to as pulse crops (faba bean, chickpea, pea, lentil and lupin), play an important role in farming systems worldwide (Halila et al. 1990; Kelley et al. 1997). Their seed protein content is high ranging from 22% in pea to 45% in lupin, and they are used for human and animal consumption in the southern and northern hemispheres. Most of the genotypes of food and feed legume species are characterised by an indeterminate growth habit: the reproductive structures are not initiated at the same time along the stem, but flowers and pods develop continuously on the plants. As a consequence, all plant organs (vegetative aerial parts, nodules, roots, reproductive organs) are competing for assimilates at any given time.

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Pulse crops are infected by fungal diseases such as grey mould or chocolate spot, ascochyta blights, anthracnose, powdery and downy mildews and rusts. The relative importance of these diseases and their effect on yield vary among countries. However, ascochyta blights affect large areas in many countries where pulses are cultivated and cause considerable losses in seed quality and quantity. In crops seeded in the autumn and winter, a practice associated with significantly higher seed yield due to the crop flowering and producing seeds in a cooler and more humid environment during the second part of the crop's life, ascochyta blights can be of particular importance. These diseases which can occur on all above-ground parts of the plant are the main foliar disease on grain legumes except for lupin.

The primary objective for this review is to highlight similarities and differences in the epidemiology across the species and across countries. The intention is to promote thinking across systems and to explore the potential of adapting successful research strategies from one agricultural system to another, and from one *Ascochyta* spp. to other species of this genus.

The pathogens

All pathogens responsible for ascochyta blights belong to the genus *Ascochyta*, with the different species *pisi* and *pinodes* (on pea), *rabiei* (on chickpea), *lentis* (on lentil), *fabae* (on faba bean), and *viciae* (on vicia). Another closely related species is *Phoma medicaginis* var. *pinodella* which frequently occurs on pea and causes symptoms on internodes and leaves similar to those induced by *A. pinodes*. It is one of the three species implied in the ascochyta blight complex of pea (Hare and Walker 1944; Wallen 1965), which differentiates ascochyta blight of pea from that on lentil, faba bean and chickpea, all of which are caused by a single fungal species. An understanding of the interrelationship between the three pathogens of the pea ascochyta complex is still lacking, but appears to involve interspecies competition and possibly different climatic optima as well as

resistance in pea cultivars to particular species. In Australia, Bretag et al. (1995a) observed that in 436 seed lots tested 94.8% of isolates were *M. pinodes*, 4.2% *P. medicaginis* and 1.0% *A. pisi*. Skolko et al. (1954) reported that 85% of Canadian ascochyta blight-infected seed lots were infected by *A. pisi*. However, since the introduction of resistance to *A. pisi* in the mid 1960s *M. pinodes* has become the dominant species found on Canadian pea seed (R.A.A. Morrall, Department of Biology, University of Saskatchewan, Canada, pers. comm.). In France, *M. pinodes* is the dominant pea pathogen, but *A. pisi* can be found in southern France (C. Le May, INRA, France, pers. comm.). Comparison of these pathogens in terms of culture characteristics, symptoms, and disease cycle are given by Jones (1927) and Hare and Walker (1944).

Reproduction

All of the *Ascochyta* spp. produce pycnidia with hyaline, straight or slightly curved conidia (pycnidiospores), and generally with one septum except those from *P. medicaginis* var. *pinodella* which are in most cases unicellular.

The teleomorph of these fungi belong to the genera *Mycosphaerella* (*A. pinodes*) or *Didymella* (*A. lentis*, *A. rabiei*, *A. fabae*) with the exception of *A. pisi* for which the teleomorph has not been described to date (Table 1). The teleomorph of *P. medicaginis* var. *pinodella* was described as *M. pinodes* by Bowen et al. (1997), but this was not supported by subsequent morphological studies and RAPD analysis (Onfroy et al. 1999). *Mycosphaerella pinodes* is homothallic and forms its pseudothecia on the senescent stipules during the second part of the cropping season. In contrast, all *Didymella* species are heterothallic with two mating types (Wilson and Kaiser 1995; Kaiser et al. 1997; Barve et al. 2003; Peever et al. 2004) and pseudothecia, if present, develop at the end of the cropping season on dead plants. These sexual fruiting structures permit the fungi to overwinter and are considered to play an important role in generating pathogen variability (Kaiser 1997).

Table 1 Characteristics of the *Ascochyta* species infecting grain legume species

	A. pisi	*P. m.* var. *pinodella*	*A. fabae*	*A. lentis*	*A. rabiei*	*A. pinodes*
Host	Pea	Pea	Faba bean	Lentil	Chickpea	Pea
Teleomorph	Unknown	Unknown	*Didymella*	*Didymella*	*Didymella*	*Mycosphaerella*
Mating types	–	0	+	+	+	0
Chlamydospores	0	+	0	0	0	0/+

– = no sexual form

0 = absence

+ = presence

Development of the anamorph or teleomorph depends on specific environmental and/or nutritional conditions. Pycnidia are formed generally during the vegetative cycle and pseudothecia at the end of the cropping cycle on senescent tissues (Agrios 2004). Navas-Cortes et al. (1998) determined that humidity levels of 100% were important for the development of pseudothecia of *D. rabiei* whereas low temperatures (5–10°C) were critical for pseudothecial maturation. Roger and Tivoli (1996a) observed that the development of reproductive structures and sporulation of *M. pinodes* were optimal at 20°C, but were sparse at 15°C. The switch from pseudothecial development to pycnidial formation seems to occur as a response to the availability of nutrients, with pseudothecia developing under poor and pycnidia under high nutrient conditions. It was also suggested that a reduction of the phytoalexin concentration (pisatin) is involved in this switch to pseudothecial development (Roger and Tivoli 1996b; Roger et al. 1999a).

Pathogenesis of *Ascochyta* spp.

Type of parasitism

The status of ascochyta blight pathogens in regard to the type of parasitism has not been discussed in detail in the literature. *Ascochyta fabae* was found to develop intracellular hyphae in epidermal tissue of faba bean during the early stages of infection, suggesting an initial biotrophic phase (Maurin et al. 1993). Similarly, Clulow et al. (1991) observed a biotrophic phase lasting for about 48 h in pea epicotyls inoculated with *M. pinodes*. Therefore, fungi responsible for

ascochyta blights may be considered as hemibiotrophs characterised by an initial biotrophic phase that is followed by a necrotrophic phase. However, phytotoxins characteristic for necrotrophic pathogens have been isolated from the germination fluid of *A. rabiei* spores and were suggested to be of importance in early ascochyta blight development on chickpea (Höhl et al. 1991). Histological studies for the first critical 48–72 h have not been published for this pathogen to determine whether host invasion follows a biotrophic or necrotrophic strategy.

Infection process

A few studies have been published that describe the infection process by *Ascochyta* spp. on grain legumes at the microscopic level. Direct penetration of the pea cuticle by the germ tube was observed for *A. pisi* (Brewer and MacNeill 1953) and for *M. pinodes* on leaves but not on epicotyls (Clulow et al. 1992). Epicotyl penetration typically occurred through an appressorium which Roger et al. (1999a) also observed for leaf infections by this pathogen. Subsequent colonization by *A. pisi* was characterized by an initial phase of subcuticular development followed by intercellular spread which resulted in the collapse and death of the host cells, beyond which no hyphae were detected (Brewer and MacNeill 1953; Heath and Wood 1969). *Asochyta fabae* was also observed to invade intercellular spaces between epidermis and mesophyll of susceptible faba bean lines within the area of the lesion (Maurin et al. 1993). In contrast, intra- as well as intercellular hyphae in the palisade mesophyll 24 h after inoculation were visible in pea leaves inoculated with *M. pinodes* resulting in a rapid

disorganisation and browning of the contents of invaded cells and those adjacent to intercellular hyphae beyond the necrosis (Heath and Wood 1969).

The second phase of infection consists of aggressive mycelium extension which coincides with the diffusion of toxins, enzymes and/or suppressors allowing rapid fungal progression in dead tissues by removing physical barriers (toxins and enzymes) and delaying or suppressing the host defense responses (suppressors). Among toxins, enzymes and suppressors, toxins have received most attention to date, whereas less is known about enzymes and suppressors. All three types of compounds are produced by *A. rabiei* which has been studied most extensively (reviewed by Barz and Welle 1992 and Jayakakumar et al. 2005), and the toxins solanopyrones A, B and C have been isolated from culture filtrates and spore germination fluids (Höhl et al. 1991; Kaur 1995). The role of the phytotoxin ascochitine has been comprehensively studied on faba bean infected with *A. fabae* (Beed et al. 1994), and on pea infected with *A. pisi* (Marcinkowska et al. 1991). Suppressors (reviewed by Shiraishi et al. 1994) and cell wall-degrading enzymes (Heath and Wood 1971a) were isolated and characterised from *M. pinodes* on pea leaves.

Phytoalexins

Phytoalexins with activity against *Ascochyta* spp. have been identified such as medicarpin and maackiain in chickpea (Daniel et al. 1990), and pisatin in pea (Bailey 1969; Heath and Wood 1971b). Experiments have shown that resistant chickpea cultivars infected with *A. rabiei* showed a significantly higher accumulation of phytoalexins than susceptible cultivars (Weigand et al. 1986; Daniel et al. 1990). On pea, Bailey (1969) showed that pisatin concentrations decreased with plant senescence while the tissue became more susceptible to infection by *M. pinodes*, leading Roger and Tivoli (1996b) to conclude that any factor contributing to decreased pisatin concentration and accelerated plant senescence may favour the production of pseudothecia of *M. pinodes*. Roger et al. (1999a) suggested that leaf wetness possibly favours pycnidial development

by *M. pinodes* because of a dilution of pisatin. Toyoda et al. (1995) found that exogenously applied plant lectins were able to induce the production of pisatin.

In faba bean, six phytoalexins have been described with activity against germ tubes of primarily *Botrytis cinerea*, and some against *B. fabae* (Hargreaves et al. 1977), but no phytoalexin has been identified with activity against *A. fabae*.

Symptoms

Symptoms develop on all aerial parts of the plant and consist of necrotic lesions. Interestingly, the different ascochyta blight fungi induce similar symptoms except for those of *M. pinodes* and *P. medicaginis* var. *pinodella*. All other *Ascochyta* spp. cause well delineated lesions with clear borders, in the centre of which numerous pycnidia are formed. On the stems, these fungi cause deep necrotic lesions which can lead to breaking of stems and death of plant parts above the affected zone. *Mycosphaerella pinodes* initially produces small lesions in the form of numerous flecks. Leaves with many lesions wither before the lesions become large, especially on the lower portion of the plants. Stem lesions are initiated at the bases of dead leaves and spread above and below that point. They coalesce to encircle the entire lower stem which generally does not break. All species cause necrosis on pods which results in seed infection. Heavily infected seeds have more or less severe discolourations and can shrivel in the most serious cases. *Phoma medicaginis* var. *pinodella* can cause the same necrosis on foliage as *M. pinodes*, but foot-rot is the more typical symptom.

Disease prevalence and yield losses

Yield losses include both weight and quality losses due to seed infection. The difficulty to precisely determine the impact of ascochyta blights on yield is evident in the large ranges reported for yield losses in published papers. In field pea, Bretag et al. (2000) found that disease severity varied considerably between years,

regions and fields in the same region and was attributed to differences in climatic conditions and in the availability of inoculum (Bretag 1991). In chickpea, Nene (1981) quoted yield losses ranging from 10–20% to 50–70% depending upon the country and the year. Similar ranges of yield losses have been published for other ascochyta blight diseases. Very few publications list yield loss assessments as an explicit objective, and consequently there is a lack of data collected under well-characterised conditions. As a result, diseases are frequently classified as important or major with a % loss in the introduction, but without any indication of frequency of such losses, or how and under what conditions they were encountered.

A survey of 68 pea fields in Victoria (Australia) in 1986 by Bretag et al. (1995b) showed that yield losses varied from 3.1 to 26.4% with a mean of 18.1%. In field experiments, Ahmed and Morrall (1996) observed seed infections ranging between 12 and 29% among 10 lentil genotypes, between 35 and 43% among five *A. lentis* isolates, and a significant relationship between Area Under the Disease Progress Curve (AUDPC) and % seed infection. On around 150 commercial lentil seed samples collected from four states of the US Pacific Northwest between 1982 and 1985, the levels of infection with *A. lentis* ranged from 0 to 100% between years, and from 7 and 100% between three states (Kaiser and Hannan 1986). On faba bean, Gaunt and Liew (1981) reported incidence levels of *A. fabae* between 0.3 and 12% from 23 fields in New Zealand in 1977–1978 which were correlated with percentages of infected plants.

Apart from climatic factors and the availability of inoculum, yield losses or variation in seed quality also depend upon the pathogen species and the physiological stage of the plants at the time of infection. For example, in plots artificially infested with *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi*, Wallen (1965) showed that *M. pinodes* caused the greatest reduction in yield (45%). Plots inoculated with *P. medicaginis* var. *pinodella* yielded 25% lower and those inoculated with *A. pisi* 11% lower than control plots. In these plots, seed infection rates were 5.0, 15.6 and 16.6% for *P. medicaginis* var. *pinodella*,

M. pinodes and *A. pisi*, respectively. For *M. pinodes*, a relationship between yield loss and the growth stage of the pea plants at the time of inoculation was demonstrated experimentally. Xue et al. (1997) observed that *M. pinodes* reduced yield of field pea by 31, 24 and 19% in 1994, and 33, 43 and 30% in 1995 when plants were inoculated at 8–10 nodes, mid-flowering and the pod swell stages, respectively. All inoculations reduced seed weight in both years compared to the control.

Survival and primary inoculum

Four main sources of primary inoculum have been described for ascochyta blight diseases: seeds, plant debris, soil and volunteers.

Seeds

Ascochyta spp. are generally considered to be seedborne pathogens, and infected seed can be the most important source of inoculum for long distance spread. Kaiser (1997) reviewed inter- and intra-national spread of ascochyta blight pathogens of chickpea, faba bean and lentil, and showed that seeds were responsible for the introduction of various *Ascochyta* spp. into, and for dissemination within many countries worldwide. Movement of infected seed of these three crops not only leads to the introduction of virulent pathotypes, but may also spread compatible mating types into new areas which can result in the development of the teleomorph. In the case of Canada, Gossen and Morrall (1986) pointed out that seed-borne inoculum was the means by which ascochyta blights of faba bean, chickpea and lentil initially became established in this country.

The penetration of these pathogens into the inner parts of the seeds permits their survival for several years, mainly when seeds are stored at low temperatures. Corbière et al. (1994) determined that *M. pinodes* was able to survive in pea seed for four years. When pea seeds were highly infected by *M. pinodes*, the pathogen was detected in the embryo and pycnidia were observed in internal cotyledons (Moussart et al.

1998). On faba bean, Michail et al. (1983) observed that *A. fabae* could be isolated from the seed coat, cotyledons and embryo axis at the rate of 100%, 46% and 27%, respectively. *Ascochyta rabiei* was located on or in the seed coat, in a very few cases penetrated into the cotyledons, and rarely grew to the embryo. *Ascochyta lentis* was found in 36% of testae and cotelydons from seeds with more than 12% seed discolouration (Morrall and Beauchamp 1988). The mode of transmission from seed to the foliar plant parts was described as non-systemic (Dey and Singh 1994).

The rate of seed-to-seedling transmission determines how many infected seeds develop into infected seedlings and thus can influence the development of an epidemic. Pea seeds infected by *A. pisi* gave rise to only 40% infected seedlings with lesions on stems and the first two leaves, whereas almost all seedlings developing from seeds infected with *M. pinodes* showed symptoms at or below soil level (Maude 1966; Moussart et al. 1998). Low temperatures were found to increase the frequency of transmission in the case of pea (Moussart et al. 1998), but also in lentil (Gossen and Morrall 1986). Corbière et al. (1994) found seed-to-seedling transmission rates for *M. pinodes* of 100% at 15°C compared to 61.5% at 18°C and 70% at 25°C. Although seed-to-seedling frequencies are high in *M. pinodes*, Bretag et al. (1995a) found there was no correlation between the level of seed infection and the severity of ascochyta blight. This lack of correlation between incidences of seed infection and ascochyta blight severity in pea has been attributed to the importance of air-borne ascospores in the epidemiology of this disease. However, seed infection significantly influences seedling emergence.

Field experiments in lentil showed that seed infection with *A. lentis* had a significant negative effect on germination rates (Gossen and Morrall 1986). In experiments under controlled conditions, lentil seed with no visible discolouration, slight discolouration and large lesions due to *A. lentis* had seed-to-seedling transmission frequencies of 0.07, 0.21 and 0.39, respectively (Gossen and Morrall 1986).

With *A. rabiei*, Weltzien and Kaack (1984) observed that out of 95 infected seeds, 32%

produced infected plants. Surface inoculation of seed with spore suspensions of 10^9–10^{10} spores of *A. rabiei* ml^{-1} reduced germination rates to 46% compared to 99% in uninoculated seeds, and resulted in 6% healthy seedlings compared to 99% in uninoculated seed lots (Kaiser et al. 1973).

Asochyta fabae is seed-borne and splash dispersed, and the primary source of inoculum is thought to be infected seeds in commercial seed lots (reviewed by Gaunt 1983). Gaunt and Liew (1981) used six seed lots with incidences of seed infection ranging from 0.2 to 12% in field experiments and found disease incidences ranging from 2.6 to 50.0%, incidence of seed infection from 15.0 to 22.0%, and yield from 4.3 to 2.4 t ha^{-1}, respectively.

Infected stubble

In many countries, crop residue has traditionally been buried in the soil by ploughing, a procedure that generally promotes the decomposition of the stubble and thus the destruction of pathogen structures. In some countries, agronomic practices in this respect have changed dramatically, primarily to avoid soil erosion by wind after harvest, and low- or zero-till agriculture is practised where the stubble remains at the soil surface. This change in cropping practice may have had a significant impact on the importance of stubble in the epidemiological cycle in some systems. Two main cases have demonstrated the epidemiological importance of fungal survival in infected stubbles.

In Washington State, USA, the teleomorph of *A. rabiei* develops on chickpea crop residues that remain on the soil surface during winter. The number of ascospores has been estimated to reach about 15,000 mm^{-2} of tissue surface of infested stubble, and spores are released during the vegetative stage of the following chickpea crop (Trapero-Casas and Kaiser 1992a). In Spain, Navas-Cortes et al. (1995) observed that on chickpea debris left on the soil surface under natural conditions, *A. rabiei* rapidly colonized the tissues, formed abundant pseudothecia and pycnidia, and remained viable throughout the two years of their study. When plant debris was

buried, *A. rabiei* was restricted to the original lesions and lost viability within 2–4 months (Kaiser 1973). Although Navas-Cortes et al. (1995) often observed brown, thick-walled, swollen hyphae associated with fruiting structures in the infected tissues, they were unable to distinguish specialized fungal survival structures such as chlamydospores or sclerotia on either surface or buried debris as described for *A. pinodes* and *P. medicaginis* var. *pinodella* (Table 1). These structures are important allowing these species to survive in the soil for more than one year after the complete destruction of host tissues. Gossen and Morrall (1986) observed that *A. fabae*, *A. rabiei* and *A. lentis* survived at the soil surface for at least one winter season. Steep ascochyta blight gradients were modelled in lentil fields by Pedersen et al. (1993) who found that one-year old residue and volunteers present at the field border affected the new crop up to 50 m into the lentil field.

In the case of *M. pinodes* under growing conditions in Australia, pseudothecia are formed on crop residues from the previous year and the concentration of air-borne ascospores is highest in late autumn to early winter when pseudothecia mature and first become productive (Bretag 1991; Peck et al. 2001).

Soil

Few reports have investigated the behaviour of *Ascochyta* spp. in the soil. Some detailed studies have been conducted only on the species involved in the ascochyta blight complex of pea. The ability of soil cultures of *P. medicaginis* var. *pinodella* and *A. pinodes* to form chlamydospores was considered to be a major factor for their survival, whereas no chlamydospores were formed in soil cultures of *A. pisi* (Wallen and Jeun 1968). The authors observed that chlamydospores enabled these fungi to survive for at least 12 months in sterilised soils. Wallen et al. (1967) reported that *P. medicaginis* var. *pinodella* was present in most soils where peas were grown in eastern Canada, and also in some soils where peas had not been grown for one to five years. *Ascochyta pinodes*, in comparison, was isolated less frequently from these soil samples. Studying

A. pinodes survival in soil and aerial dissemination through the pea cropping season, Peck et al. (2001) observed that infection from stubble was initially high but dropped to low levels after one year, while infection from soil inoculum declined slowly over three years.

Volunteers

Volunteers have sometimes been indicated in the transmission of inoculum from field borders into the fields. The role of the volunteer plants has been well described for faba bean. For this crop, Bond and Pope (1980) found a distinct gradient of ascochyta blight-infected winter bean plants from the border to the centre suggesting that spread from volunteer plants in adjacent fields was a much more important source of infection than infected seed. However, debris from previous crops may also be important (Lockwood et al. 1985).

In general, the importance of the different sources of primary inoculum may vary depending upon the species, cropping practices and climatic factors. For example, based on the distribution of primary symptoms on upper parts of the chickpea plants, Milgroom and Peever (2003) concluded that *D. rabiei* ascospores were the dominant type of primary inoculum, and were more important than infected seeds in the northwest of the USA. In contrast, in Canadian and Australian chickpea fields, infected seeds and chickpea stubble are considered to be the primary sources of inoculum, although airborne ascospores are of relevance for long-distance dispersal (Chongo et al. 2004; Pande et al. 2005).

Disease development

Ascochyta blights are polycyclic and secondary cycles are generally due to a succession of pycnospores released from plant tissue to tissue, or from plant to plant. Researchers have generally described five to ten cycles of pycnidiospore production and re-infection during the cropping season which results in a progression of the disease along the plant from the base to the top. Leaves or leaf axils tend to be the first plant

tissues that are infected, followed by stems, pods and seeds. It is only in the case of *M. pinodes* that ascospores are involved in secondary cycles of infections.

Different phases of the disease cycle

The epidemiological development of ascochyta blights can be considered as a race between the host plant which grows and develops depending on environmental conditions, and the pathogen which may infect the plant at the early stage of development at the base of the plant and from there continues to spread to upper plant parts as they develop. As a consequence, for some crops like faba bean and pea, ascochyta blight severity is generally very high on the lower parts of the plants, but can be very low on the uppermost parts where plant tissue has escaped infection until that time.

Maurin and Tivoli (1992) described this epidemiological pattern in three phases for winter faba bean starting with the first lesions caused by *A. fabae* until seed infection has occurred:

(1) Disease initiation is characterised by the appearance of a few lesions on seedlings during the winter. The fungus spreads from plant to plant and disease incidence increases rapidly. The cold, wet weather during crop establishment is usually favourable to pathogen development on slow-growing seedlings.

(2) The subsequent phase of plant infection starts at the end of the winter when plants begin to grow more rapidly. Lesions, initially limited to the foliage, develop on stems and spread to the top of the plant. The epidemic spreads on foliage from node to node. Disease severity may be reduced when the climatic conditions are unfavourable for spreading (very little rainfall) while the number of foliar nodes increases with plant growth. Hence, plants may escape a severe attack temporarily because plant growth is significantly faster than the progression of the pathogen. During this second epidemic phase, the spread of the fungus strongly depends on rainfall, but it is very likely that disease severity during this phase also depends on inoculum pressure, a consequence of the initial infection.

(3) During the pod infection phase the pathogen infects those pods that develop on infected nodes. Conidial dispersal by splashing during rain facilitates the contamination of the lowest pods. This third phase takes place when plant growth slows down after the flowering stage, and during pod initiation and filling. At this growth stage of the plant, even light disease pressure can induce severe damage because pod infection causes infection of the seeds.

This epidemiological cycle can be generalised for different legume crop species. Regarding the respective roles of ascospores and pycnidiospores, three scenarios of increasing complexity are described among the grain legume species considered.

In the first scenario (Fig. 1), the epidemic is exclusively based on successive pycnidiospore cycles. This scenario is characteristic for epidemics caused by *A. pisi*, *A. lentis*, *A. fabae*, *P. medicaginis* var. *pinodella* and *A. rabiei* (under certain growing conditions). Primary inoculum can be infected seeds (for all of these species), infected debris and in some cases chlamydospores in the soil, or ascospores (for *A. fabae, A. lentis* and *A. rabiei*). For some of the species, the sexual form has been identified but the epidemic role of ascospores is not evident or unknown in the epidemiological cycle. For example, in ascochyta blight on lentil, the commonly described disease cycle observed in Canadian lentil fields is only based on pycnidiospore dispersal during the cropping period and ascospores are not present (Morrall 1997). In contrast, in Australia (Galloway et al. 2004) and in the northwest of the USA (Kaiser and Hellier 1993), the teleomorph has been detected in the field on lentil straw. Similarly, Porta-Puglia (1990) described *A. rabiei* epidemics for the Mediterranean basin caused by pycnidiospores which are dispersed by rainfall and intensified by wind. However, the life-cycle of *A. fabae* on faba bean described by Jellis and Punithalingam (1991) includes the teleomorph *D. fabae*.

Fig. 1 Disease cycle of ascochyta blight (*Ascochyta fabae*) on faba bean
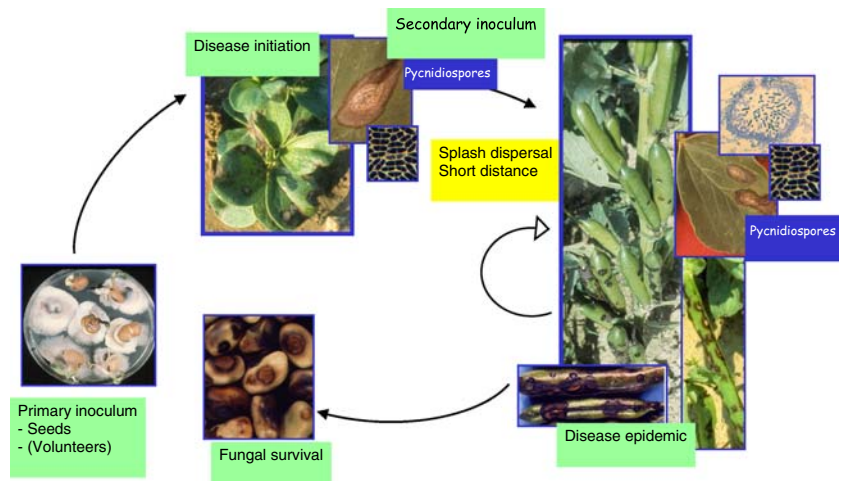
In the second scenario (Fig. 2) the epidemic is based on both successive ascospore discharges as primary inoculum and successive pycnidiospore cycles. This scenario has been described for epidemics caused by *A. rabiei* and *M. pinodes* when infected stubble remains at the soil surface. Pseudothecia are formed on dead tissues at the end of the cropping season and can constitute an important source of primary inoculum by successive discharges in the following crop at the end of winter and during spring. Trapero-Casas et al. (1996) and Peck et al. (2001) found that ascospores were trapped mostly during winter and spring, thus confirming that this is the period of pseudothecial maturation.

For ascochyta blight on chickpea, Trapero-Casas and Kaiser (1992a) pointed out the important role of ascospores in the disease cycle in the Palouse region of eastern Washington and northern Idaho, USA and stressed the necessity to consider ascospores on chickpea straw. To conserve moisture and reduce soil erosion in those regions, infested chickpea debris remains on the soil surface, thus favouring the development of pseudothecia of *D. rabiei* during the fall and winter months. The first vegetative period of crop development was shown to overlap with the second half of ascospore release. Kaiser (1997) described the life cycle of ascochyta blight of chickpea where both pycnidia and pseudothecia may develop on overwintered chickpea debris.

Fig. 2 Disease cycle of ascochyta blight (*Didymella rabiei*) on chickpea
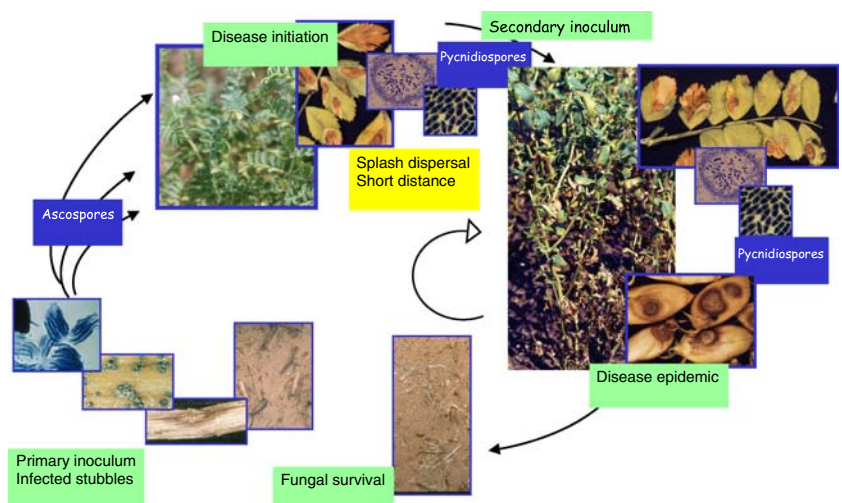
In the third scenario (Fig. 3), the epidemic is based on both ascospores discharges as primary and secondary inoculum, and successive pycnidiospore cycles. This scenario describes epidemics caused by *M. pinodes*. Primary inoculum consists of ascospores in addition to infected seed and plant debris. However, pseudothecia are also formed alongside pycnidia during the cropping season; consequently ascospores are released during the entire season and constitute an important source of secondary inoculum.

Roger and Tivoli (1996b) showed that the disease cycle of *M. pinodes* started with the dissemination of ascospores after which pycnidia developed rapidly in lesions on stipules, on green plant tissue or on senescent tissue. The number of pycnidia was highly correlated with disease severity. Pycnidiospores dispersed by rain splash are responsible for secondary infections over short distances and further increases in disease severity, thus accelerating tissue senescence. As a consequence of this early tissue senescence, an early production of pseudothecia is initiated which are only produced on senescent tissues. This also explains why pseudothecia are present predominantly at the bases of pea plants. Their role seems to be essential in the epidemic because they contribute to increased inoculum concentration and disease severity, and thus accelerate the epidemic cycle. After rainfall, pseudothecia

release ascospores which are dispersed over long distances by wind. In trials, pycnidiospores were principally trapped in the first 20 cm above the soil surface whereas ascospores were also trapped above the crop canopy. The formation of fruiting bodies progresses from the base to the top of the plants during crop development. Frequently, pycnidia and pseudothecia are simultaneously present on the same stipule. This is unusual because the sexual stage most commonly follows the asexual stage in plant pathogenic fungi (Agrios 2004).

Environmental and climatic factors

Temperature and moisture after inoculation are probably the two key environmental factors in the epidemiology of ascochyta blights on legumes and have a major influence on the length of the incubation and latent periods.

Under optimal temperatures and leaf wetness, the incubation period for *M. pinodes* was shown to be 1–2 days (Roger et al. 1999a), 5 days for *A. pisi* (Heath and Wood 1969) and *A. rabiei* (Trapero-Casas and Kaiser 1992b), and 6 days for *A. lentis* (Pedersen and Morrall 1994). The latent period for *M. pinodes* was 3–4 days (Roger et al. 1999a), 5–6 days for *A. rabiei* (Trapero-Casas and Kaiser 1992b), 6–7 days for *A. lentis* (Pedersen and Morrall 1994), 8–10 days for

Fig. 3 Disease cycle of ascochyta blight (*Mycosphaerella pinodes*) on pea
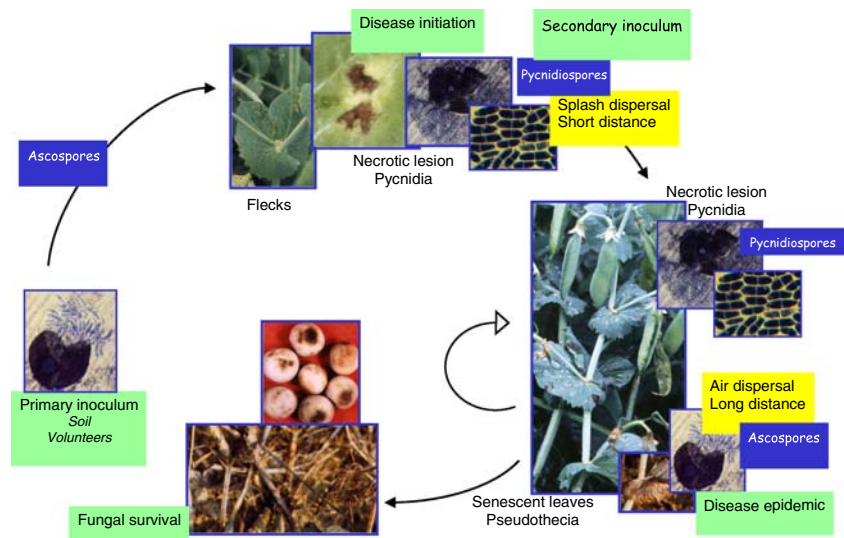
A. fabae (Wallen and Galway 1977) and 10 days for *A. pisi* (Heath and Wood 1969).

In lentil, temperature had little effect on lesion size and number of pycnidia per lesion, but infection frequency was higher at 10 and 15°C than at 25°C (Pedersen and Morrall 1994). Trapero-Casas and Kaiser (1992b) observed that at 20°C, 7.6 and 17 h of wetness were required for *A. rabiei* to cause light and severe infection, respectively. At temperatures lower or higher than 20°C, significant infection was only observed after longer periods of wetness. At the optimum temperature of 20°C, pycnidiospores of *M. pinodes* germinated within 2 h, appressoria developed after 6 h and the penetration peg invaded the leaf after 8 h (Roger et al. 1999a). This process was slowed down or stopped as a result of intermittent dry periods (Roger et al. 1999b).

Spore dispersal

It has been recognized that spore dispersal has a major impact on the onset and development of epidemics. Pycnidiospores are dispersed by water-splashing that restricts their spread to short distances, except in cases where fine water droplets containing spores are picked up by air currents and transported over longer distances. However, long-distance spread in general occurs when airborne ascospores are produced and moved by air currents and wind.

Pycnidiospore release by splashing is the common characteristics of spore dispersal for all the ascochyta blight pathogens. This phenomenon has been well described for ascochyta blights of lentil, faba bean, chickpea and pea. For example, Pedersen et al. (1993) found that although less than 50% of pycnidiospores of *A. lentis* were spread further than 15 cm from their place of origin, and only very few to distances up to 70 cm, strong winds could carry them to over distances as far as 100 m. The movement of spores in aerosol droplets was described by Pedersen and Morrall (1995) to explain greater horizontal spread of ascochyta blight when strong winds occurred during rainfall. This led Morrall (1997) to the conclusion that wind had a major influence on the spread of ascochyta blight in lentil despite the absence of air-borne ascospores. On pea, Roger

and Tivoli (1996b) showed that pycnidiospores of *A. pinodes* were dispersed by rain-splash to a maximum of 30 cm above the soil surface, with the result that this zone had highest infection levels.

Ascospore release has mainly been described for *D. rabiei* and *M. pinodes*. Trapero-Casas and Kaiser (1992b) reported that new infection foci in chickpea fields were often located at distances of 10–15 km from the nearest chickpea field infected with ascochyta blight suggesting airborne ascospore dissemination as primary inoculum. Spore trapping revealed that ascospores were trapped on rainy days during daylight, and 70% of those were trapped between 12:00 and 18:00 h. This observation was confirmed by Gamliel-Atinsky et al. (2005) who used chickpea plants as living traps in the field to show that ascospore dispersal was dependent on successive rain and dry periods. Extensive spore-trapping experiments in peas infected with *M. pinodes* revealed that the majority of ascospores was captured within the canopy and only a small proportion escaped beyond the boundaries of the canopy (Roger and Tivoli 1996b). This demonstrated that as the canopy develops it becomes a barrier to ascospore dispersal, probably because air circulation in the canopy is reduced.

Cultural factors and host resistance

With the exception of stubble disposal and fungicide applications, which will not be discussed here further, few data have been published on the role of cultural factors such as seeding date and plant density on ascochyta blight development.

Early seeding of Australian pea crops in May (late autumn) resulted in higher infection rates of plants and seeds by *M. pinodes* and lower yields compared to late-seeded peas in July because of higher levels of primary ascospore inoculum and longer periods of leaf wetness (Bretag et al. 1995a, 2000). Similar observations were reported for *A. rabiei* on chickpea (Trapero-Casas et al. 1996). On pea, Tivoli et al. (1996) demonstrated that seed densities of 30, 60, 90 and 120 seeds m^{-2} resulted in percentages of infected stem by *M. pinodes* of 34.7, 41.8, 50.1 and 53.7%, and yield losses of 24.7, 37.4, 40.6 and 46.4%, respectively.

Host resistance is considered to have the greatest effect on ascochyta blight epidemics. Although poorly understood, pulse crops appear to become more susceptible to infection by *Ascochyta* spp. with increasing plant age. For example, *M. pinodes* was shown to be more aggressive on old tissues which was correlated with decreasing phytoalexin concentrations in the tissue (Heath and Wood 1971b). Similarly, partially resistant chickpea cultivars were shown to lose resistance to *A. rabiei* with increasing plant age (e.g., Trapero-Casas and Kaiser 1992b; Singh and Reddy 1993; Chongo and Gossen 2001), but it was also suggested that newly developed plant tissue on these plants showed higher resistance than older tissue (Chongo and Gossen 2001). In lentil, in contrast, Pedersen and Morrall (1994) observed that tissues below the top four or five nodes on the main stem and secondary branches were almost completely resistant suggesting that resistance increased as tissue matured.

Studying the effect of resistance in lentil on the infection process of *A. lentis*, Pedersen and Morrall (1994) and Ahmed and Morrall (1996) found that although the incubation periods were similar among lentil cultivars, AUDPC, the number of lesions, lesion length and width and the number of pycnidia per lesion were negatively correlated with the level of resistance in the cultivars. Similar observations have been made on pea and faba bean (Maurin and Tivoli 1992; Prioul et al. 2003).

Differences in plant architecture have also been implicated in influencing ascochyta blight epidemics by modifying the microclimate in the canopy. For example, faba bean stem length was found to be negatively correlated with pod infection by *A. fabae* and moisture levels of the soil surface, and pods higher up on taller stems escaped infection more readily than pods on short stems (Jellis et al. 1985). Resistance to *M. pinodes* was found to be positively correlated with lodging resistance, and both lodging and mycosphaerella blight were negatively correlated with the proportion of xylem, lignin and fibre content of pea stems (Banniza et al. 2005). Le May et al. (2005) developed a simulation model for the growth of pea infected with mycosphaerella blight by incorporating architectural features such as stem height, branching ability and lodging resistance into the model.

Modelling

Modelling has the objective to formalize by means of mathematical equations the knowledge on disease epidemiology. This leads either to a description of all or some segments of an epidemic with the objective to understand its mechanisms, or to forecast the risk of appearance or development of the disease in relation to factors like temperature, rainfall, and crop rotations. Models can therefore become an important and integral part of Integrated Disease Management (IDM). As a first step, disease epidemics have often been described in terms of temporal and spatial models to describe disease progress. More complex models have been separated into two groups, mechanistic models and empirical models. Mechanistic models are based on a concept or hypothesis about the mechanism of the interaction and therefore require prior knowledge of the variables involved. Empirical models, in contrast, do not require prior knowledge about the variables and look at the best mathematical fit of the model to the data, e.g., temperature, rainfall, and crop rotation. Both types of models have been used to describe diseases of grain legumes.

Quantitative descriptions of temporal and spatial developments of ascochyta blights are few. A comprehensive study of temporal and spatial dynamics of mycosphaerella blight in Canada was published by Zhang et al. (2004). Disease progress in space and time was well described by logistic models. Steepest disease gradients were identified upwind at the end of the growing season. Geostatistical analysis of spatial patterns revealed differences in disease severity depending upon geographic directions attributed to different wind speed and direction.

For ascochyta blight on lentil, Pedersen and Morrall (1994) established regression equations which predict incubation and latent periods at different temperatures. Using a mechanistic approach, ascochyta blight severity of chickpea was described as a function of temperature and of

the natural logarithm of the length of the wetness period, predicting that approximately 20°C was the optimum temperature requiring the shortest wetness period (Trapero-Casas and Kaiser 1992b). For severe infection, a minimum of 7.6–10.3 h of wetness was required at a temperature range of 15–25°C based on this model. Similarly, polynomial equations were used to predict incubation period, latency of *M. pinodes* and ascochyta blight severity on pea based on temperature and wetness period (Roger et al. 1999a).

Using and building upon a disease-coupled crop growth model published by Béasse et al. (2000), Le May et al. (2005) developed an improved model to predict the impact of ascochyta blight in pea on yield components by incorporating a combination of disease progression in the canopy (number of nodes affected by the disease) and the structure of the canopy (leaf area index profile). For doing so, they first estimated the contribution of each node to radiation absorption, then calculated the reduction in contribution of each node due to disease and finally combined the individual contributions which allowed them to estimate crop growth. Using data from six varieties they showed a good fit between estimated and observed values.

It is surprising to observe the low number of forecasting models for ascochyta blights on grain legumes. The few forecasting models that have been described only permit prediction of one or two phases of the epidemic, such as disease appearance, disease development, fungal reproduction or spore release. For *M. pinodes* on pea, Salam et al. (2006) established a model using historical weather data to forecast disease development under different weather scenarios. The prediction of ascospore release proved to be critical in determining seeding dates in order to avoid spore deposition on the newly establishing crop. Using data from fifteen pea cultivars, Bretag et al. (1995b) developed empirical crop loss models that allowed yield loss to be estimated based on disease severity.

Gamliel-Atinsky et al. (2005) confirmed that pseudothesial formation and maturation of *D. rabiei* required low temperature and moisture periods, based on which in combination with other published data Shtienberg et al. (2005)

developed an empirical model to forecast pseudothecial maturation.

Conclusion

This review on the epidemiology of the various *Ascochyta* spp. in pulse crops highlights common and specific features of the different ascochyta blights. The common features can be summarized as (1) all species produce pycnidia, (2) except for *A. pisi*, the teleomorph is present, (3) the role of infected seed as primary inoculum is significant, (4) they form the same types of symptoms except for *M. pinodes* and *P. medicaginis* var. *pinodella*, (5) plant compounds like phytoalexins and pathogen toxins appear to be involved in the host-pathogen interactions, (6) ascochyta blights are polycyclic diseases and epidemics develop on leaves, stems, pods and finally seeds, (7) temperature and moisture are the two primary environmental factors affecting disease development, (8) pseudothecia generally are formed at the end of the cropping cycle (except for *M. pinodes*) and are implied in inoculum survival. Species-specific features are (1) the type of symptoms caused by *M. pinodes*, (2) the length of incubation and latency periods which are different among the different ascochyta blights, (3) the role of infected stubble as primary inoculum in some parts of the world, (4) the involvement of pseudothecia of *M. pinodes* as secondary inoculum.

Reviewing the literature revealed several areas where there is a clear lack of data:

(1) The exact involvement of the soil as primary inoculum is unclear, but we can expect that molecular detection tools for pathogens from soil will improve our knowledge.

(2) The timing of primary inoculum deposition on the crop is difficult to assess and techniques other than trap plants and spore traps are lacking to easily estimate inoculum quantities above the plant canopy.

(3) There is a general lack of understanding of the host-pathogen interactions, at the microscopic level to some degree, but more so at the biochemical and molecular levels. With the exception of *A. rabiei*, the majority of papers, in particular, on biochemical aspects

of the systems are 20–40 years old, and it can be expected that modern tools of molecular biology and biochemistry could have a significant impact on our ability to investigate these host-pathogen systems.

(4) Information on the role of cultural factors on ascochyta blight epidemics is sparse in the published literature. Surprisingly little information has been published that could improve integrated disease management of these diseases.

(5) Mechanistic modelling for life-cycles of all the pathogens and for the epidemics during the cropping period is missing.

(6) More forecasting models are required that establish simple relationships between climatic and epidemic events and that are easy to implement.

When comparing the well-researched areas in ascochyta blight epidemiologies with those where there are obvious gaps in our knowledge, it becomes obvious that the latter are of equal importance, but appear to have been neglected because of lack of research concepts, tools or resources. Clearly, several of these areas would benefit greatly from the use of molecular tools and the application of modern statistical methodology. It is also apparent that some aspects of ascochyta blight research was conducted decades ago, and although still of considerable importance, would benefit from reassessments using modern tools and techniques. On the other hand, some more traditional research areas seem to have been neglected almost completely in favour of molecular research. For example, it seems surprising that to date there is insufficient data to answer the fundamental question of whether *Ascochyta* spp. are hemiobiotrophs or necrotrophs. There seems to be an urgent need to boost new research initiatives in the area of epidemiology of ascochyta blights which combine traditional epidemiological strategies with new tools provided by molecular biology and biochemistry to elucidate the mechanisms of these host–pathogen interactions. Knowledge and data of that nature are essential to make progress in the development of quantitative mechanistic models, but will also assist in resistance breeding

by providing an understanding of the complexity of the interaction. Beyond that, there is an obvious gap in applied research which investigates the role and sources of primary inoculum and cultural factors to develop and improve current integrated disease management strategies.

Research on the epidemiology of *A. rabiei* and *M. pinodes* is most comprehensive and advanced, and may serve as an example and an inspiration for the other species as well as for each other. In *M. pinodes* the most detailed information has been gathered on disease development, the initiation, location and dispersal of various dispersal structures, and the effect of disease development on the plant in terms of photosynthetic activity as well as yield formation. A comprehensive review specifically on the epidemiology and control of ascochyta blight on field pea was recently published by Bretag et al. (2006). Research on this species has greatly benefited from concentrated long-term studies primarily in France and Australia whereas research efforts on other species has been either more limited in scope due to limitations in resources (e.g., *A. lentis*), or has been scattered across various countries, diverse climates and cropping systems which has hampered the transfer and application of research results in a more comprehensive manner (e.g., *A. rabiei*). However, research on the advance of *M. pinodes* in, and its interaction with the host at the microscopic, biochemical and molecular level is sketchy. Ascochyta blight of chickpea, in comparison, is much better understood in those areas whereas comprehensive field epidemiological studies comparable to those conducted for *M. pinodes* are lacking. It can be speculated that filling those gaps for both of these pathogens may lead to major advances in disease management: a better understanding of the host-pathogen interaction of *M. pinodes* on pea may give fresh impetus to the breeding of resistant pea cultivars which appears to have stagnated, but which could significantly simplify asochyta blight control in this crop. On the other hand, a more comprehensive understanding of the epidemiology of *A. rabiei* could result in better disease management strategies that are urgently needed in countries like Canada and Australia. The elegant disease

forecasting system developed and utilized in Israel (Shtienberg et al. 2005) could be a starting point for studies in other countries to clarify whether, to what degree and under which conditions ascospores induce ascochyta blight on chickpea. This would then determine whether the underlying model could be adapted and adopted elsewhere to prevent primary infection of the chickpea crop by targeted fungicide sprays to kill the ascospores early in the season. It is obvious that in systems where infected seed, stubble, pycnidia and potentially ascospores can initiate ascochyta blight, disease forecasting is bound to become more complex as exemplified by the models developed for *M. pinodes* in France (Béasse et al. 2000; Le May et al. 2005). It is probably safe to say that for those systems we may not know enough about the relative importance of each of these sources, which may be highly variable depending upon the location and year. It is unlikely that the prevention of primary infection can be achieved in such systems; hence models are required which describe the entire life-cycle of these pathogens in response to environmental factors. Also, some of the species are thought to occur and cause damage in the anamorphic phase only, but experiences with *A. rabiei* and *A. lentis* in various parts of the world have shown that a focused attempt has to be made to truly determine whether ascospores can be excluded from the life-cycle of these organisms.

Among world crops, grain legumes play a minor role and consequently research on these crops and their pathogens is bound to be restricted by fewer resources and researchers. This, in combination with the diversity of skills and knowledge required to tackle those gaps in ascochyta blight epidemiology outlined here, should present a strong incentive for future international collaborations.

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FULL RESEARCH PAPER

Development of ascochyta blight (*Ascochyta rabiei*) in chickpea as affected by host resistance and plant age

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Abstract Ascochyta blight caused by *Ascochyta rabiei*, is the most destructive disease in many chickpea growing countries. Disease development varies with the growth stage and host resistance. Hence, disease development was studied in cvs ICCX 810800 (resistant), ICCV 90201 (moderately resistant), C 235 (moderately susceptible), ICCV 96029 and Pb 7 (susceptible) under controlled environment (ICRISAT, Patencheru) and field conditions (Dhaulakuan, Himachal Pradesh) at seedling, post-seedling, vegetative, flowering and podding stages. Under controlled environment, the incubation period and terminal disease reaction (TDR) did not vary significantly at different growth stages against virulent isolate AB 4. Cultivars ICCX 810800, ICCV 90201 and C 235 showed a significantly longer incubation period than the susceptible cv. Pb 7. Cultivar ICCX 810800 showed slow disease progress and the least TDR. Field experiments were conducted during the 2003–2004 and 2004–2005 growing seasons. During 2003–2004, TDR was higher in plants inoculated at podding and the flowering stage and the lowest disease reaction was recorded in ICCX 810800. A severe epidemic during 2004–2005 was attributed to the favourable temperature, humidity and well distributed high rainfall. TDR did not differ significantly at any of the growth stages in susceptible cvs ICCV 96029 and Pb 7. With respect to seeding date and cultivar, the highest yield was recorded in the early-sown crop (1,276.7 kg ha^{-1}) and in ICCV 90201 (1,799.3 kg ha^{-1}), respectively. The yields were greatly reduced in all the cultivars during 2004–2005 and the highest yield was recorded in ICCX 810800 (524.7 kg ha^{-1}). Integrated disease management using resistant cultivars, optimum sowing period and foliar application of fungicides will improve chickpea production. The experiment under controlled environment and field conditions (during the epidemic year) showed a similar disease development.

Keywords *Ascochyta rabiei* ·
Cicer arietinum · Disease dynamics ·
Plant growth stage · Resistance

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Introduction

Chickpea is world's third most important grain legume. It is a major source of dietary protein and a significant contributor to agricultural sustainability by fixing atmospheric nitrogen. It diversifies agricultural production systems in rotation with cereals. During the year 2004–2005, the world chickpea production was approximately 8.58 million tonnes from an area of approximately 11.16 million hectares (Ikisan 2000). The seed yield varies from <390 to 3,600 kg ha^{-1} depending upon environmental conditions and management for biotic and abiotic constraints. Ascochyta blight, caused by *Ascochyta rabiei*, is a major factor in the low productivity of chickpea in various countries of western Asia and north Africa, the northwestern plains in the Indian subcontinent, Australia, North America, Latin America and southern Europe (Gan et al. 2006; Nene and Reddy 1987; Pande et al. 2005). It infects during all growth stages of plants where temperature and rainfall are favourable for pathogen development (Pande et al. 2005, Shtienberg et al. 2000) and may cause yield losses up to 100%. The disease can be managed by the cultivation of resistant cultivars. Plant age had been reported to have no impact on disease resistance in some cultivars (Trapero-Casas and Kaiser 1992) whereas, in others it has been reported to decline with plant maturity (Chongo and Gossen 2001; Gan et al. 2006; Nene and Reddy 1987; Singh and Reddy, 1993). This change from resistance to susceptibility with maturity refutes the importance of resistance as the main strategy for managing this disease. In this context, present studies were undertaken to study development of ascochyta blight as affected by plant age, environmental factors and resistance status of some Indian cultivars.

Materials and methods

Host

Five desi chickpea cultivars; C 235, ICCV 90201, ICCX 810800, ICCV 96029 and Pb 7 were used in the present studies. The pedigree, origin and resistance status of these cultivars is given in Table 1. Cultivar Pb 7, an old cultivar from Punjab (India) and ICCV 96029 were highly susceptible to ascochyta blight. Cultivar ICCV 96029 is a very early maturing and cold tolerant line suitable for contingent crop planning in the northwestern plain and hill zone of India. ICRISAT lines ICCX 810800 and ICCV 90201 have been released in Himachal Pradesh (India) for cultivation as ascochyta blight and Fusarium wilt-resistant cultivars. Cultivar C 235 is an old and widely adapted variety released in many countries.

Pathogen

Single conidial isolates of *A. rabiei*, AB 4 (isolated from infected plants at Hissar, Haryana) and isolate AB 6 (isolated from infected plants at Dhaulakuan, Himachal Pradesh) were used for the controlled environment and field studies, respectively. Isolate AB 04 was highly virulent whereas, isolate AB 06 was moderate in virulence (Basandrai et al. 2005). The isolates were multiplied on chickpea dextrose agar medium for 15 days and used for the studies.

Controlled environment studies

The experiment was conducted in the growth chambers at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru.

Table 1 Pedigree, origin and resistance status of chickpea cultivars

Cultivar	Pedigree	Origin	Reaction to ascochyta blight
ICCX 810800	GL 769 × ILC 202	ICRISAT	Resistant
ICCV 90201	GL 769 × ICC 1069	ICRISAT	Moderately resistant
C 235	C 1235 × IP 58	PAU, Ludhiana	Moderately susceptible
ICCV 96029	ICCV 2 × ICCV 93929	ICRISAT	Highly susceptible
Pb 7	ICC 4991	A local selection from Punjab	Highly susceptible

Plant growth conditions

Plants of the test cultivars were raised in 25 cm diameter plastic pots filled with a mixture of sterilized sand and vermiculite (10:1), in a greenhouse maintained at 25 ± 3°C and a 12–13 h photoperiod under natural light. Staggered sowing was done for 8 weeks to produce plants that were 2–9 weeks of age representing five distinct growth stages (Table 2) at the time of inoculation. Five plants were maintained in each pot with three replications.

Inoculation and incubation

The pots with plants of different growth stages were transferred to the growth chamber maintained at 20 ± 1°C and light intensity of 1,500–1,600 lux using artificial daylight fluorescent tubes. The inoculum was mass-multiplied on Kabuli chickpea seeds. Seeds were soaked overnight in water and about 50 g of these seeds were transferred in 250 ml flasks. These were sterilized by autoclaving at 121°C (15 psi) for 25 min. Highly sporulating inoculum of the isolate AB 4, grown on chickpea dextrose agar, was transferred aseptically onto the seeds in the flask. The inoculated flasks were incubated at 20 ± 0.5°C with a 12 h alternate light and dark period. The flasks were frequently shaken to avoid clumping of inoculum. Abundant conidial production was obtained after 6–8 days. The conidia were harvested in sterilized water. The plants were inoculated by spraying a suspension of isolate AB 4 (5×10^4 conidia ml^{-1}) in water. The inoculated plants were allowed to dry for 4 h and thereafter incubated at 100% continuous RH for 6–7 days.

Table 2 Growth stage of chickpea cultivars at which inoculations were done

Age in weeks	Growth stage	Growth stage description
2	I	Seedling
3	II	Post-seedling: Branch initiation
4–5	III	Vegetative: Branching continue- Floral bud initiation
6–7	IV	Flowering: Flowering and stem hardening
8–9	V	Podding: Flowering to pod formation

Data recording

The plants were observed daily to determine incubation period i.e. the period (days) from inoculation to appearance of first visible symptoms. Thereafter, the data were recorded for disease reaction on alternate days for each plant in the pot on a 1–9 scale (Nene et al. 1981). These data were used to determine the dynamics of disease progress.

Field studies

Field trials were conducted in the experimental fields at the Choudhary Saravan Kumar Himachal Pradesh Agricultural University, Hill Agricultural Research and Extension Centre, Dhaulakuan, India, a hot spot for ascochyta blight, during 2003–2004 and 2004–2005. The test cultivars were planted in 0.9×3 m plots with row-to-row and plant-to-plant spacing of 30 and 10 cm, respectively in a split-plot design, with date of sowing as the main plot and varieties as sub-plots. Genotype ICCV 96029 was also included in the field studies. The first planting was done on 24 October during both years and subsequently, four more staggered plantings were done fortnightly to produce plants at five different growth stages, viz. seedling (I), post-seedling, branch initiation (II), vegetative (III), flowering (IV) and podding stage (V). The plots were inoculated by frequently spraying conidial inoculum of isolate AB 6 (10^6 conidia ml^{-1}), mass-multiplied on Kabuli chickpea seeds, starting 4–6 weeks after the last seeding when the plants of all growth stages were available. It was repeated at four-day intervals. In all, 4–5 inoculations were carried out. Ascochyta blight-infected debris was also broadcast in each plot along with the first spray to encourage uniform development of the disease and to prevent disease escape. A Perfo-spray system was used to provide humidity on the dry days between 11.00 h and 17.00 h for 20–30 min every 3 h.

Data recording

The data were recorded on 10 randomly selected plants for terminal disease reaction (TDR) on 1–9 scale (Nene et al. 1981) and yield (kg ha^{-1}) during both years. TDR was also assessed at 2, 4, 6, 8, 10

and 12 weeks after inoculation during 2004–2005 and was used to determine the dynamics of disease progress. Analyses of variance were done using the CPCS1 computer programme.

Results

Controlled environment studies

The data recorded for incubation period and TDR under controlled environmental conditions are given in Tables 3 and 4.

Incubation period

The incubation period on the susceptible cv. Pb 7 was the shortest among the cultivars in the trial (3.0 days). Incubation period in cvs ICCX 810800, ICCV 90201 and C 235 was statistically longer compared with the susceptible cv. Pb 7. Cultivar ICCX 810800 showed the longest incubation period (6.4 days), significantly longer than the moderately resistant (ICCV 90201)

and moderately susceptible (C 235) cultivars. The incubation period of the test cultivars did not differ significantly among the different growth stages.

Dynamics of disease development

The disease progress in the test cultivars at different growth stages is presented in Figs. 1–4.

In cv. C 235, the slowest disease progress was recorded in plants inoculated at flowering stage followed by plants inoculated at the post-seedling stage (Fig. 1). In cv. ICCX 810800, the plants at the seedling stage recorded the slowest disease progress (Fig. 2). In cv. ICCV 90201, the slowest disease progress was recorded in plants inoculated at the post-seedling stage followed by plants inoculated at the seedling stage (Fig. 3). The dynamics of disease progress in cv. Pb 7 was similar at all the growth stages (Fig. 4).

Terminal disease reaction

Cultivars ICCX 810800, C 235, ICCV 90201 and Pb 7 developed a TDR of 6.8–8.1, 8.0–8.7, 7.2–8.5 and 8.5–9.0, respectively in plants inoculated at different

Table 3 Effect of growth stage and cultivar on incubation period of ascochyta blight infection under controlled environmental conditions

Cultivar	Incubation period (days) at growth stage					Mean
	I	II	III	IV	V	
C 235	4.00	4.00	4.33	4.33	4.33	4.20
ICCX 810800	6.67	6.67	6.33	6.67	5.67	6.40
Pb 7	3.00	3.00	3.00	3.00	3.00	3.00
ICCV 90201	4.00	4.33	4.00	4.67	5.33	4.47
Mean	4.42	4.50	4.42	4.67	4.58	
CD (5%)	Cultivar = 0.52, Plant age = NS, Cultivars × Plant age = NS					

Table 4 Terminal disease reaction of chickpea cultivars against *A. rabiei* inoculated at different growth stages under a controlled environment

Cultivar	Disease reaction (1–9) at growth stages					Mean
	I	II	III	IV	V	
C 235	8.7	8.4	8.0	8.3	8.3	8.3
ICCX 810800	6.8	7.3	7.5	7.1	8.1	7.3
Pb 7	9.0	8.8	8.7	8.5	8.8	8.8
ICCV 90201	7.3	7.2	8.3	7.6	8.5	7.8
Mean	8.0	7.9	8.1	7.9	8.4	
CD (5%)	Cultivar = 0.44, Plant age = NS, Cultivars × Plant age = NS					
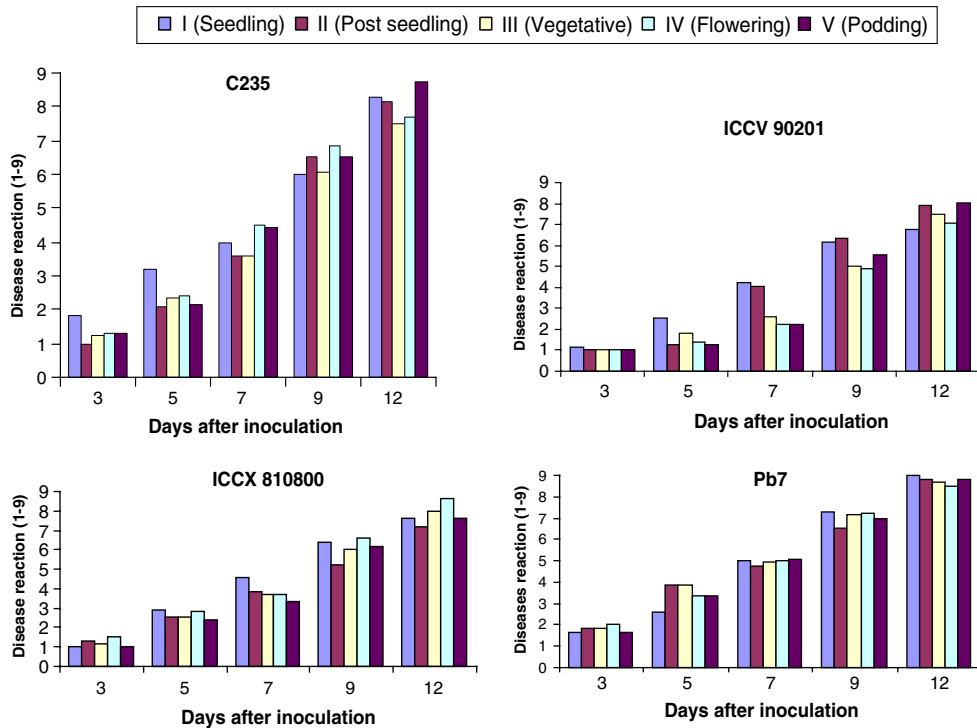
Figs. 1–4 Dynamics of disease development against *Ascochyta rabiei* isolate AB 04 in chickpea cvs C 235, ICCV 90201, ICCX 810800 and Pb 7 at different growth stages (I Seedling;

II Post-seedling; III Vegetative; IV Flowering and V Podding stage) under controlled environmental conditions at ICRISAT, Patencheru

growth stages (Table 4). The mean TDR was non-significant among plants inoculated at different growth stages, but it differed significantly among cultivars. Cultivar ICCX 810800 developed the lowest TDR (7.3) followed by ICCV 90201 (7.8). ICCX 810800 showed the lowest (6.8) TDR at the seedling stage. The resistant cv. ICCX 810800 and the moderately resistant cv. ICCV 90201 had a longer incubation period, slower disease development and the least TDR in plants inoculated at the younger stage and thus showed rate-reducing resistance.

Field studies

Blight appeared in epidemic form during 2004–2005 and it was moderate during the 2003–2004 growing season. All of the cultivars developed the lowest TDR in plants inoculated at the seedling to vegetative stage and the TDR increased consistently in plants at later growth stages (Table 5) during the 2003–2004 growing season. With regard to cultivar averaged

over growth stages, the highest and the lowest TDR were recorded in cvs ICCV 96029 (6.1) and ICCX 810800 (2.2), respectively. With regard to growth stage averaged over cultivars, the highest and the lowest TDR values were recorded in the plants inoculated at the podding stage (5.3) and the seedling stage (2.9), respectively. In cv. ICCX 810800, TDR was the highest (4.4) in plants inoculated at the flowering stage and it differed significantly from plants inoculated at other growth stages. During the 2004–2005 growing season, the TDR was not statistically significant with respect to growth stage and the cultivar × growth stage interaction. However, the TDR differed significantly among cultivars. The highest TDR was recorded in cv. Pb 7 (8.9) followed by ICCV 96029 (8.8) and, averaged over the growth stages, cv. ICCX 810800 showed the lowest TDR (2.9) followed by ICCV 90201 (4.3).

The effect of ascochyta blight on yield of chickpea cultivars in plants inoculated at different growth stages are summarized in Table 6. In general, the

Table 5 Terminal disease reaction (TDR) of *Ascochyta rabiei* on chickpea cultivars inoculated at varying growth stages under field conditions at Dhaulakuan during 2003–2004 and 2004–2005

| Cultivar	Terminal disease reaction (1–9) on plants inoculated at growth stages											
	2003–2004						2004–2005					
	I	II	III	IV	V	Mean	I	II	III	IV	V	Mean
C 235	2.6	2.6	3.3	4.4	4.4	3.5	6.5	5.5	5.7	6.3	5.3	5.9
ICCV 90201	2.1	2.6	2.6	3.2	3.7	2.8	4.2	4.6	4.0	4.6	4.3	4.3
ICCV 96029	4.8	4.8	5.7	6.4	8.8	6.1	8.8	9.0	8.1	9.0	8.9	8.8
ICCX 810800	1.2	1.3	2.0	4.4	2.2	2.2	1.8	2.9	3.1	3.2	3.7	2.9
Pb 7	3.9	4.0	3.9	6.8	7.5	5.2	9.0	9.0	8.7	9.0	8.7	8.9
Mean	2.9	3.1	3.5	5.0	5.3		6.1	6.2	5.9	6.4	6.2	
CD (5%)	Cultivar = 0.48, Growth stage = 0.65, Cultivar × growth stage = 1.1						Cultivar = 0.5, Growth stage = NS, Cultivar × growth stage = NS					

yield was higher during 2003–2004 as compared with the epidemic year 2004–2005. Averaged across the inoculation treatments at various growth stages, the highest yield was recorded in moderately resistant cv. ICCV 90201(1,799.3 kg ha^{-1}) followed by C 235 (1,259.5 kg ha^{-1}). Averaged across cultivars, the highest yield (1,276.7 kg ha^{-1}) was recorded in the earlier-sown crops (inoculated at the podding stage) and yield decreased consistently with delay in the sowing (Table 6). However, in the very early cv. ICCV 96029, the highest yield (600 kg ha^{-1}) was recorded in late sown crop (inoculated at the post-seedling stage). Yield for this inoculation treatment was similar to that of the crop inoculated at the vegetative stage (563 kg ha^{-1}).

During the 2004–2005 growing season, the highest yield was recorded in cv. ICCX 810800 (524.2 kg ha^{-1}) averaged across sowing dates and in crops sown earlier and inoculated at the podding stage, when averaged across cultivars. In cvs ICCX 810800 and ICCV 90201, the highest seed yield was obtained in the earlier-sown crop (1,204.8 and 307.0 kg ha^{-1}, respectively). Yield decreased drastically in the delayed sowings. Negligible yield was obtained from the susceptible cvs Pb 7 and ICCV 96029.

The dynamics of disease development in cultivars inoculated at different growth stages during 2004–2005 are shown in Figs. 5–9. In cv. C 235, disease appeared earlier and progressed faster in plants

Table 6 Effect of ascochyta blight infection on yield (kg ha^{-1}) of chickpea cultivars sown at different dates at Dhaulakuan during 2003–2004 and 2004–2005

Cultivar	Yield (kg ha^{-1}) in plants inoculated at growth stage											
	2003–2004						2004–2005					
	I	II	III	IV	V	Mean	I	II	III	IV	V	Mean
C 235	1,251.9	1,084.1	1,353.0	856.7	1,751.9	1,259.5	89.6	20.7	18.9	57.4	254.4	88.2
ICCV 90201	1,107.4	1,203.7	1,024.8	2,723.3	2,937.0	1,799.3	168.1	83.7	174.1	232.6	307.0	193.1
ICCV 96029	444.4	600.0	563.0	113.7	53.7	355.0	7.8	7.8	32.6	71.9	23.3	28.7
ICCX 810800	64.1	555.6	387.8	1,254.4	1,281.5	708.7	130.0	130.0	368.9	787.4	1,204.8	524.2
Pb 7	37.0	403.7	340.7	74.1	359.3	243.0	7.8	7.8	0	7.8	7.4	6.2
Mean	581.1	769.3	733.7	1,004.4	1,276.7		80.7	50	118.9	231.4	359.4	
CD (5%)	Cultivar = 40.0, Growth stage = 37.8, Cultivar × growth stage = 84.4						Cultivar = 19.7, Growth stage = 10.7, Cultivar × growth stage = 43.0					
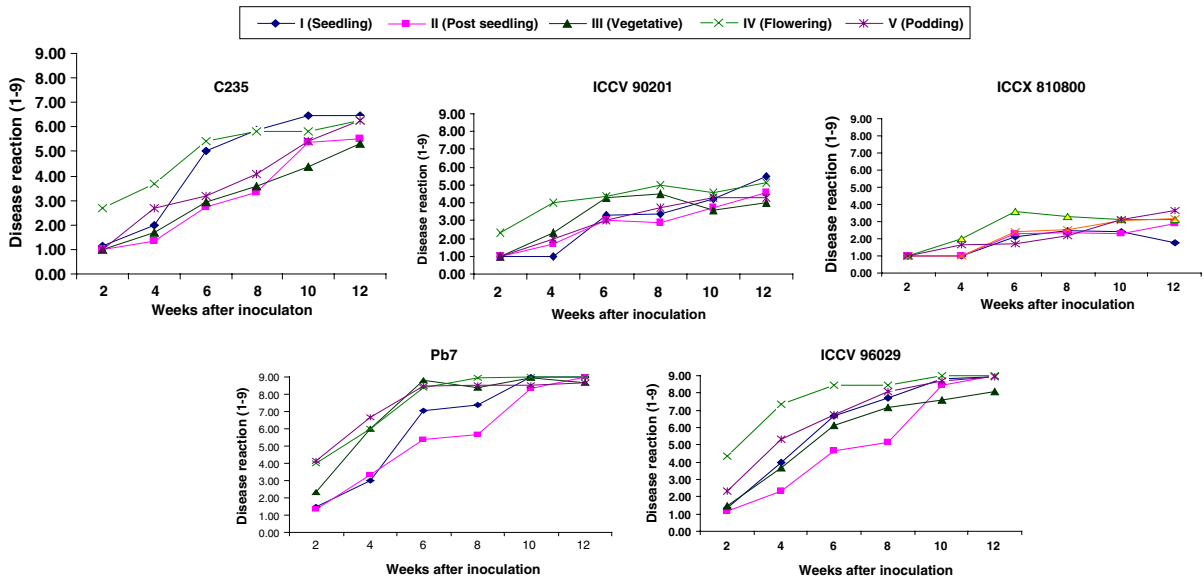
Figs. 5–9 Dynamics of disease development against *Ascochyta rabiei* isolate AB 06 in chickpea cvs C 235, ICCV 90201, ICCX 810800, Pb 7 and ICCV 96029 at different growth stages (I Seedling; II Post-seedling; III Vegetative; IV Flowering and V Podding stage) under field conditions at Dhaulakuan
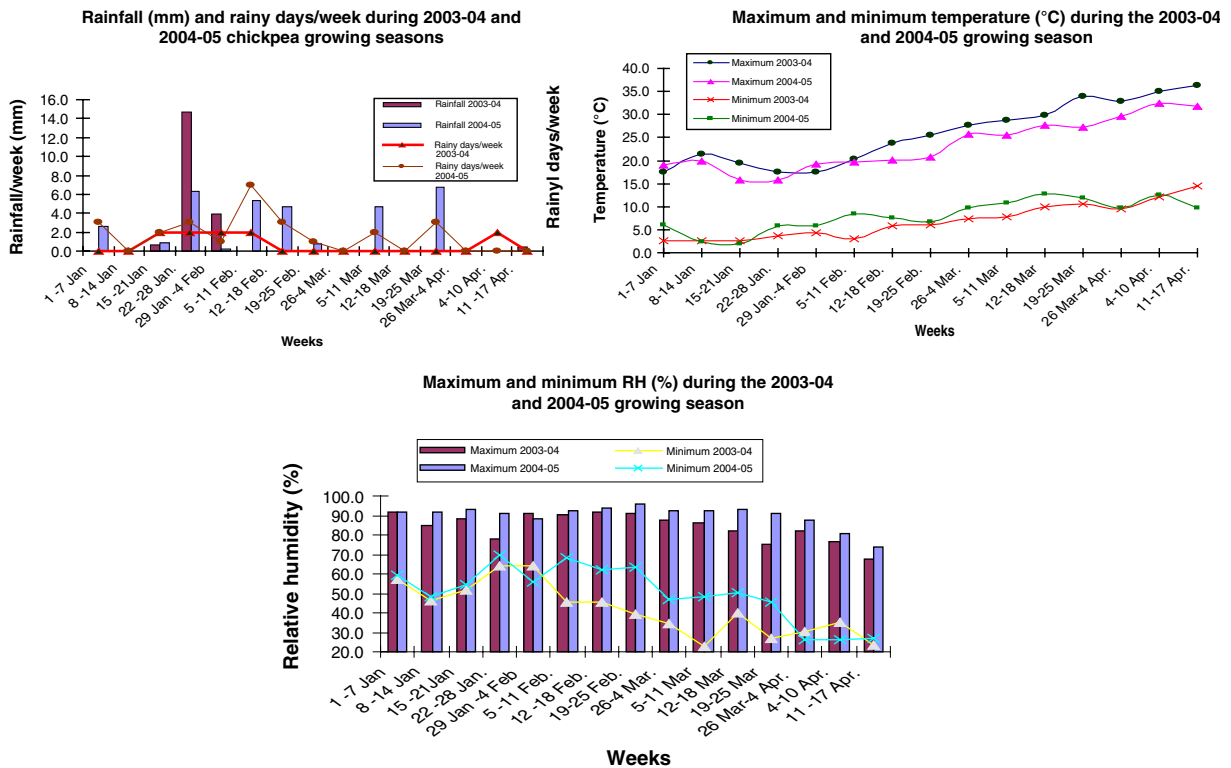
inoculated at flowering, followed by plants inoculated at the seedling stage (Fig. 5). In ICCV 90201, the disease appeared earlier and progressed faster in plants inoculated at the flowering stage, followed by plants inoculated at the podding stage (Fig. 6). In susceptible cultivars, symptoms appeared 2 weeks after inoculation for treatments inoculated at the vegetative stage or later, and 4 weeks after inoculation for plants inoculated at the seedling or post-seedling stages. In contrast, symptoms in cv. ICCX 810800 appeared 4 weeks following inoculation of plants at the vegetative and podding stages and at 6 weeks following inoculation of plants at the seedling, post-seedling and flowering stages (Fig. 7). The disease progressed at a faster rate in plants inoculated at the podding and vegetative stages and progressed at the slowest rate in plants inoculated at the seedling stage.

In susceptible cvs ICCV 96029 and Pb 7, the disease appeared earlier and progressed more quickly at all growth stages, with a TDR of 8.1–9.0 (Fig. 8 and 9).

Discussion

The effect of growth stages on development of ascochyta blight was studied in cultivars with varying

levels of resistance under controlled environment and field conditions. Under the controlled environment conditions, symptoms developed earlier in susceptible cv. Pb 7 with an incubation period of 3.0 days. The incubation period was statistically longer in resistant (ICCX 810800), moderately resistant (ICCV 90201) and moderately susceptible (C 235) cultivars. It was the least at podding stage in cv. ICCX 810800. The incubation period in moderately resistant cv. ICCV 90201 and moderately susceptible cv. C 235 also differed significantly compared with the susceptible cv. Pb 7. Similarly, TDR was also statistically the lowest in cv. ICCX 810800 and it was numerically lower at the seedling stage. This may be because in resistant cultivars, old tissues become more vulnerable to infection than new growth (Chongo and Gossen 2001). Cultivar ICCX 810800 showed a high level of resistance at the seedling to vegetative stage which declined at the flowering to podding stage under controlled environment and field conditions during the epidemic year. These results support earlier studies (Chongo and Gossan 2001; Nene and Reddy 1987; Singh and Reddy 1993) that showed increased ascochyta blight susceptibility as the plant matured. The increased susceptibility in older plants of resistant cv. ICCX 810800 may be due to developmental gene expression, as resistance genes may be highly

Rainfall (mm) and rainy days/week during 2003-04 and 2004-05 chickpea growing seasons

Maximum and minimum temperature (°C) during the 2003-04 and 2004-05 growing season

Maximum and minimum RH (%) during the 2003-04 and 2004-05 growing season

Figs. 10–12 Maximum and minimum temperature (°C); rainfall intensity (mm) and distribution (rainy days/standard week) and mean maximum and minimum RH (%) during the cropping season 2003–2004 and 2004–2005

expressed during the seedling to vegetative stage rather than at maturity. This differential response of resistance at different growth stages may be due to the increased secretion of maleic acid (Singh and Sharma 1998), activity of enzymes namely chitinase and exo-chitinase (Nehra et al. 1997), phytoalexins, namely medicarpin and maackianin and their biosynthetic bioenzymes, lytic protein enzymes and other PR proteins (Hanselle and Barz 2001).

Plant growth stage had no effect on disease progress and TDR in highly susceptible cvs Pb 7 and ICCV 96029, and these were severely blighted at all growth stages under controlled environment and field conditions during epidemic year 2004–2005. These results were supported by earlier studies (Chongo and Gossen 2001; Trapero-Casas and Kaiser 1992) that showed that growth stage had no effect on disease development in susceptible cultivars.

In the field experiments, substantial differences were observed in TDR among the test cultivars. Characteristic symptom expression, pycnidial fruiting bodies in concentric rings, was more pronounced in adult plants (8–9 weeks-old) in the field whereas in the growth chamber and in plants at an earlier stage the disease appeared as water-soaked lesions.

During the year 2003–2004, the moderately resistant cv. ICCV 90201 gave the highest yields in the earlier-sown crop and declined with the delay in sowing. This supported earlier studies that showed early-sown moderately resistant cultivars produced a 15–300% higher yield than those sown late (Gan et al. 2002; Siddique and Sedgley 1986). This may be because sowing at the optimum time resulted in the maximum use of available resources and the plants were subjected to fewer stresses (Gan et al. 2002; Siddique and Bultynck 2004). Regardless of blight infection, delayed sowing resulted in lower grain yields as delayed sowing may not have allowed adequate grain filling prior to crop maturity (Gan et al. 2006). In contrast, yield of cv. ICCV 96029 increased with the delay in sowing and the highest yield was obtained when the crop was sown in mid-December. ICCV 96029 is a super early cultivar which flowered in 50–52 days. The earlier-sown crop

(sown 24 October 2003) flowered by mid-December, when the minimum temperature was <5°C, which resulted in lower pollen viability and embryo abortion, leading to poor pod setting (Basandrai et al. 2005), whereas the late-sown crop flowered by mid-February and thus escaped low temperature stress resulting in optimum flowering and pod setting.

During the epidemic year 2004–2005, resistant ICCX 810800, moderately resistant (ICCV 90201) and moderately susceptible (C 235) cultivars produced much lower yields compared with that obtained in 2003–2004. Though the yield level was comparatively lower in the resistant cv. ICCX 810800, it still gave the highest yield (1,204.8 kg ha^{-1}) in the early-sown crop, and then declined with the delay in sowing. No grain yield was obtained in highly susceptible cvs Pb 7 and ICCV 96029. This supports earlier results (Chongo et al. 2000a, b; Gan et al. 2006; Shtienberg et al. 2000) that showed under cool and wet conditions, application of foliar fungicides is required to realize optimum yield and quality even in resistant cultivars.

The low TDR during the year 2003–2004 may be attributed to the low weekly mean rainfall (0.7–>15 mm over 3 weeks) against 0.17–6.74 mm over 9 weeks during the season (Fig. 10).

During 2003–2004 growing season, the average minimum and maximum temperature remained below 5°C and 21.5°C, respectively until 11 February. Subsequently, minimum and maximum temperature varied from 6 to >10°C and 23.8 to >30°C and 9.4 to 14.4 and 32.3 to 36.9°C from 12 February to 18 March and 19 March to 17 April, respectively. The maximum temperature varied from 15.8 to >21°C from 1 January to 25 February, 21 to >28°C from 26 February to 25 March and was below 33°C from 17 March to 17 April 2005. The minimum temperature varied from <5°C to >13°C during the growing season except during the period 8–21 January, when it was around 2°C (Fig. 11). It is evident that during the 2004–2005 growing season, maximum temperatures were favourable for disease development, and even the minimum temperature was higher and more favourable compared with the 2003–2004 growing season. During the 2004–2005 growing season mean maximum RH was <90% during 11 out of 15 weeks of active disease development, in contrast to only 5 weeks during 2003–2004 growing season (Fig. 12). Furthermore, the mean weekly minimum RH, 45.5–

68.4% during the period 5 February–25 March, 2005 was higher compared with 22.6–45.7% during the same period in the 2003–2004 growing season (Fig. 12). Temperatures of 20 ± 1°C, RH of >90% and leaf wetness of 17 h are optimum for the infection, development and spread of ascochyta blight (Pande et al. 2005, Trapero-Casas and Kaiser 1992). In addition, leaf wetness periods greater than 8-days results in the production of higher numbers of pycnidia and conidia on infected leaves (Jhorar et al. 1997). Such favourable conditions were prevalent in the controlled environment at ICRISAT and during the year 2004–2005 at Dhaulakuan, which led to severe disease development. Jhorar et al. (1997) observed that increased dry periods immediately after inoculation resulted in reduced disease severity and low disease development. Hence, low disease levels during the 2003–2004 growing season may be attributed to the continuous dry spell.

Blight severity in the controlled environment was higher and more consistent than under field conditions; this was because isolate AB 04 was more virulent than AB 06 (Basandrai et al. 2005) and environmental conditions were highly favourable and less variable than under field conditions.

The resistant and moderately resistant cultivars showed rate-reducing residual resistance against the virulent isolate AB 4, expressed as longer incubation periods, slower disease development and lower TDR. The highly resistant cv. ICCX 810800 and highly susceptible cvs Pb 7 and ICCV 96029 showed the same trend for ascochyta blight development at different growth stages under controlled environment and field conditions during the epidemic year. Hence, growth chamber and field screening under epidemic conditions at hot spots like Dhaulakuan are equally effective and may compliment each other.

All the cultivars used in the present study were developed in India, where *A. rabiei* is highly variable in virulence (Basandrai et al. 2005; Nene and Reddy 1987; Pande et al. 2005; Singh and Sharma 1998). Under such conditions, growing susceptible cultivars, namely Pb 7 and ICCV 96029, can result in total crop loss and even resistant cultivars such as ICCX 810800 can suffer heavy losses (Chongo and Gossen 2001; Chongo et al. 2000b; Pande et al. 2005). Efforts are being made to popularise chickpea cultivation in north western India. It will result in a substantial increase in the area grown to the crop. High levels of

resistance are not available against all pathotypes of *A. rabiei* in cultivated chickpea (Basandrai et al. 2005; Nene and Reddy 1987; Pande et al. 2005; Singh and Sharma 1998). Resistant cultivars such as ICCX 810800 still show reduced resistance at the flowering stage. Hence, for the successful cultivation of chickpea, integrated management of ascochyta blight using available resistant cultivars, disease-free seed and need-based foliar application of fungicides will be the practical option.

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FULL RESEARCH PAPER

Assessment of airborne primary inoculum availability and modelling of disease onset of ascochyta blight in field peas

**Alexandra Schoeny · Stéphane Jumel ·
François Rouault · Christophe Le May ·
Bernard Tivoli**

Abstract Ascochyta blight is a serious disease
affecting field peas. In France, disease management
relies mainly on scheduled chemical applications
without taking into account the actual disease risk. A
better understanding of the factors affecting disease
onset would therefore help in the timing of the first
application. Field experiments involving eight sowing
dates between mid-September and mid-December
were conducted for two consecutive years. The
seasonal dynamics of airborne inoculum were inves-
tigated through trap plants. The weekly availability of
airborne primary inoculum was extremely low during
autumn and winter and was partially influenced by
mesoclimatic conditions. Disease onset occurred
between mid-October and early March depending on
the sowing date. Generally, the later the sowing date,
the longer the period between sowing and disease
onset. This was due to an increase in the period
between sowing and emergence. Disease onset was
observed 14–35 days after emergence. A disease onset
model based on the calculation of weather-dependent
daily infection values ($DIVs$) was established, assum-
ing that disease onset occurs once the temperature and
moisture requirements for incubation are met. Cumu-
lative daily infection values ($cDIVs$) were determined
by sowing date and experiment through addition of
consecutive $DIVs$ between emergence and disease
onset. A frequency analysis of $cDIVs$ was performed
to determine the 10th and 90th percentiles of the
distribution. An analysis of the observed and pre-
dicted values showed that observed disease onset
dates were almost always included in the forecast
window defined by these two percentiles. This study is
the first attempt to predict ascochyta blight onset in
field peas and should contribute to development of a
more rational fungicide application strategy.

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Introduction

Ascochyta blight is a serious disease affecting field
peas in most pea-growing regions of the world,
particularly in the temperate areas of Europe, North
America, Australia, and New Zealand (Bretag and
Ramsey 2001). The disease, mainly caused by

Mycosphaerella pinodes, infects all aerial organs of the plant (leaves, stems, flowers, pods) and can cause yield losses of up to 75% when conditions are favourable for an epidemic (Lawyer 1984). The disease affects yield either indirectly through reduction of biomass production (Béasse et al. 2000; Garry et al. 1998; Le May et al. 2005), or directly through pod infection (Béasse et al. 1999). The relative importance of these two effects depends on the location of the symptoms on the plant and therefore on the precocity and intensity of the epidemic. In a context of poor plant resistance, as is the case for the ascochyta blight/pea pathosystem, epidemic precocity depends mainly on inoculum availability and weather conditions.

As a polycyclic disease, ascochyta blight is initiated by primary inocula and develops by means of secondary inocula. Primary inoculum sources are numerous: infected seeds, soil, infected pea stubble, volunteer plants or legume weeds. Airborne inoculum is the most important source of primary inoculum in established pea-growing areas (Bretag and Ramsey 2001). It is mainly constituted by ascospores of *M. pinodes*, produced in pseudothecia on infected pea stubble left on the soil surface after harvest, and dispersed by wind for over 1.6 km (Lawyer 1984). Salam and Galloway (2005) developed a weather-based model to predict the seasonal release of ascospores of *M. pinodes* in Western Australia to help farmers decide when is the best time to sow their pea crops in order to avoid periods of ascospore release peaks. However, this model is limited to areas where infected pea stubble is left on the soil surface and where this primary inoculum source is obvious. In France, where pea stubble is usually removed before ploughing, little is known about the availability of airborne primary inoculum and it is therefore difficult to predict disease onset. As a consequence, disease management relies mainly on scheduled chemical applications without taking into account disease risks. For spring peas (sown between mid-February and mid-April), this fungicide application strategy is based on a first application at the beginning of flowering (BF), followed by a second application at BF + 15 days, and possibly by a third application at BF + 25 days. For winter peas (sown from mid-October to mid-January), extension services can provide little information to farmers and recommend an ''early'' application ''in case of early first symp-

toms'' without further precision (ARVALIS 2003). A better understanding of primary inoculum availability and a prediction of disease onset would therefore be particularly useful to position the first application.

The objectives of this study were (i) to characterise the pattern of airborne inoculum availability, (ii) to investigate the relationship between airborne primary inoculum availability and mesoclimate, (iii) to study the impact of sowing dates on disease onset, and (iv) to propose a predictive model of disease onset.

Materials and methods

Field experiments

Experiments were conducted during the 2003–2004 and 2004–2005 cropping seasons in Le Rheu (48°06′00″N, 1°48′00″W, 30 m above mean sea level), western France. The two adjacent fields chosen for these trials were: (i) similar in pedoclimatic environments and (ii) void of soilborne inoculum due to a rotation without pea crops during the previous five years. The experimental design consisted of eight 30 m² (3 m wide × 10 m long) plots sown with winter field peas (cv. Cheyenne, 80 seeds m^{-2}) at two-week intervals from mid-September to mid-December (Table 1 and Fig. 1). Cheyenne is susceptible to ascochyta blight and is currently the most cultivated winter field pea cultivar in France. Plots were sown perpendicularly to the prevailing wind direction (N-NW), the first plot being sown in the downwind. A 3 m wide buffer strip of bare soil was maintained between each plot to prevent cross-contamination of plots due to rain splash dispersal of inoculum.

Trap plants

Inoculum availability was assessed indirectly through trap plants. Each week from mid-September to mid-March (2003–2004) or mid-May (2004–2005), trays containing 20 trap plants (5-leaf Cheyenne pea seedlings) were placed at 1 m from the four corners of the trial (Fig. 1). After seven days of exposure, trap plants were incubated in a dew chamber (12 h-photoperiod, 20°C night/day, 100% relative humidity (RH)) for four days. The amount of viable spores deposited on trap plants was estimated as the number

Table 1 Sowing and emergence dates in field trials conducted in France in 2003–2004 and 2004–2005

Year	Sowing number	Sowing date	Emergence date
2003–2004	S1	15/09/03	29/09/03
	S2	29/09/03	13/10/03
	S3	13/10/03	03/11/03
	S4	27/10/03	17/11/03
	S5	07/11/03	01/12/03
	S6	24/11/03	05/01/04
	S7	08/12/03	19/01/04
	S8	19/12/03	02/02/04
2004–2005	S1	15/09/04	27/09/04
	S2	27/09/04	11/10/04
	S3	11/10/04	25/10/04
	S4	25/10/04	15/11/04
	S5	08/11/04	06/12/04
	S6	22/11/04	03/01/05
	S7	06/12/04	10/01/05
	S8	20/12/04	17/01/05

of resulting lesions (small, purplish-black, irregular flecks) on the five lower stipules of the plants after incubation. A mean number of lesions per plant was calculated per tray and per week. Preliminary experiments based on the method proposed by Onfroy et al. (2007) confirmed that after four days of incubation the five lower stipules of a Cheyenne pea plant all exhibited the same susceptibility to ascochyta blight (data not shown).

Emergence and disease assessment

Emergence, defined as the stage when 50% of the plants had their first true leaf emerged, was dated in

most of the cases through regular assessment of the plots (Table 1). If missed, the emergence date was estimated with an ad hoc quadratic relationship derived from the trial data:

$$y = -4.08x^2 + 57.36x + 97.49 \quad (R^2 = 0.91) \qquad (1)$$

where y is the emergence date in degree-days (from 0°C) since sowing and x is the sowing rank (from 1 to 8) (Fig. 2).

Disease was assessed at 1–2 week intervals on ten plants sampled at random in each plot. Disease onset was defined as when at least one lesion was observed on 50% of the assessed plants.

Weather data

An automatic weather station was set up near the trial plots. Air temperature and RH were measured with a temperature and humidity probe (HMP45AC, Vaisala, www.vaisala.com), precipitation was measured with a tipping bucket rain gauge (ARG100, Campbell Scientific Inc., www.campbellsci.com), and wind speed and direction were measured with a wind monitor (05103, RM Young, www.youngusa.com). Sensors were placed at 1.40–2.20 m above ground level. The data logger (CR10X, Campbell Scientific Inc.) scanned the sensors every 10 s and stored the 15-min averages.

Model conception

The ascochyta blight onset model is based on the calculation of weather-dependent daily infection values (DIVs). This method derives from initial work of Shane and Teng (1983) and further refined by Wolf
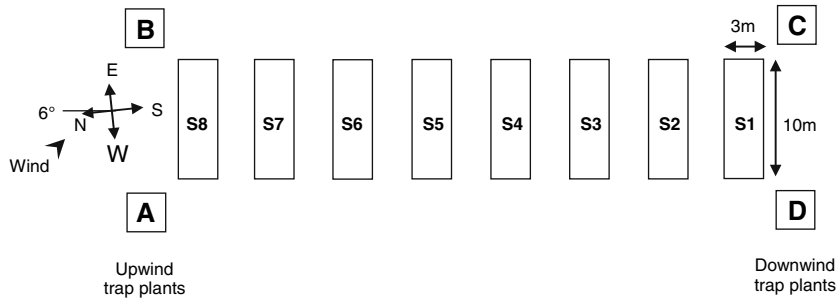
Fig. 1 Schematic representation of the experimental design used in field experiments conducted in France in 2003–2004 and 2004–2005. Eight pea plots were sown between mid-September (S1) and mid-December (S8). Trap plants were placed at 1 m from the field experiment. A and B are upwind trap plants. C and D are downwind trap plants. The arrow indicates the prevailing wind direction (N-NW)
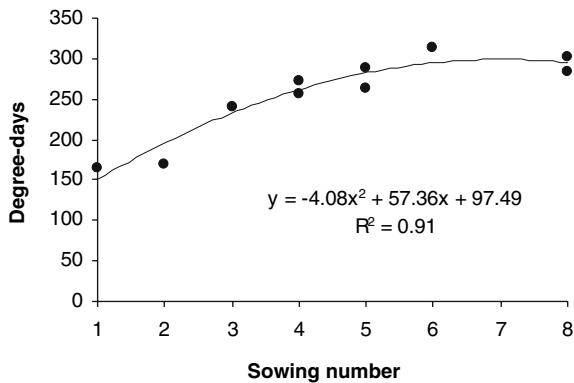
Fig. 2 A quadratic model predicting the time (expressed in degree-days from 0°C) required for emergence of the first leaf for sowing dates ranging between mid-September and mid-December in field trials conducted in France in 2003–2004 and 2004–2005

and Verreet ([2005](#)) on *Cercospora beticola* infection prediction. This modelling approach is suitable for any fungal epidemic initiated by airborne primary inocula. *DIVs* were calculated using hourly data from the automatic weather station. The effects of temperature and moisture on incubation period (time elapsed between infection and the appearance of the first symptom) were quantified through the calculation of two specific indices. *DIVs* were computed as the average of hourly values of the product of these two indices:

$$DIV_i = \frac{1}{24} \sum_{j=1}^{24} M_{ij} \times T_{ij} \in [0;1] \text{ (dimensionless)} \quad (2)$$

where DIV_i is the infection value for day i ranging between 0 (no fungal growth) and 1 (optimal growth), M_{ij} is the moisture index for day i and hour j, and T_{ij} is the temperature index for day i and hour j. M_{ij} and T_{ij} were based on published data obtained from artificial inoculation of pea plants under controlled conditions (Roger et al. [1999a, b](#)).

M_{ij} is a binary variable that expressed the capacity of the environment to provide satisfactory moisture conditions for incubation. Roger et al. ([1999a](#)) showed that leaf wetness (free water on the leaf surface) or high RH was required for infection. From these results, M_{ij} was set to 1 when rainfall was ≥0.2 mm (resolution of the rain gauge) or when RH was greater than a threshold τ:

if rainfall ≥ 0.2 mm or
RH >τ % , $M_{ij} = 1$ (dimensionless)   (3a)

otherwise, $M_{ij} = 0$ (dimensionless)   (3b)

Rather than setting τ to an arbitrary level, we tested seven threshold values: 65, 70, 75, 80, 85, 90 and 95%. From the analysis of the performance of the corresponding models, the best threshold value was then determined (see below).

T_{ij} was expressed as the ratio between the length of the incubation period at optimal and at observed temperatures. We assumed that all infection processes (germination, appressorial formation, penetration) stopped at 0°C and thus set the T_{ij} value to zero when temperature was equal to or below 0°C. T_{ij} was calculated as follows:

if $t \leq 0°C$, $T_{ij} = 0$ (dimensionless)   (4a)

if $t > 0°C$, $T_{ij} = \left(\frac{IP_{opt}}{IP_t} \right)_{ij} \in]0;1]$ (dimensionless)

(4b)

where IP_{opt} is the length of the incubation period at optimum temperature and IP_t is the length of the incubation period at temperature t.

The shortest incubation period obtained at optimum temperature (15–25°C) is one day (Roger et al. [1999b](#)). At temperatures less favourable to pathogen growth, IP_t is described by a quadratic function of temperature t and varies according to the moisture regime (leaf wet or not). In the absence of leaf wetness sensors or of leaf wetness simulation models, leaf surface was assumed to be wet when rainfall was ≥0.2 mm (resolution of the rain gauge):

$IP_{opt} = 1$ (in days)   (5a)

if rainfall ≥ 0.2 mm,
$IP_t = 0.0171t^2 - 0.6457t + 6.8$ (in days)   (5b)

otherwise, $IP_t = 0.0307t^2 - 1.195t + 12.1$ (in days)

(5c)

Although the domain of validity of the equations proposed by Roger et al. ([1999b](#)) was 5–30°C, extrapolation of this model for temperatures in the

range of 0–5°C was based on results of Hare and Walker (1944). Using radial expansion as a criterion for measurement of the effect of temperature on the mycelial growth of *M. pinodes* on potato dextrose agar plates, these authors showed that 8 days after inoculation, there was no fungal growth at 0°C and 36°C; at 4°C, the diameter of the colony was 10 mm, compared to 60–70 mm at optimum temperature. They also assumed positive fungal growth between 0 and 4°C.

For each value of RH threshold τ, cumulative daily infection values (*cDIVs*) were determined for each sowing date by adding *DIVs* between emergence and disease onset:

$$cDIV = \sum_{i=1}^{n} DIV_i \qquad (6)$$

where *cDIV* = cumulative daily infection value, i = ith day of the calculation period, and DIV_i = weather-dependent DIV for day i.

For each τ, a frequency analysis of *cDIV* values was performed to determine the 10th and 90th percentiles (respectively named *P10* and *P90*). These values define a climatic window that contains 80% of *cDIV* values. In probabilistic terms, this indicates that there is an 80% chance that disease onset will occur within the defined climatic window. For the seven values of τ tested and for each experimental situation (year × sowing date), we compared the observed disease onset date to the forecast window defined by an early date (corresponding to the *P10* value of *cDIV*) and a late date (corresponding to the *P90* value of *cDIV*). In cases where disease onset occurs outside the forecast window, it is more detrimental if it occurs before the forecast window than after. The deviations of observed from predicted values were computed separately for the cases where disease onset occurred before and after the forecast window:

if $DO_{obs} < DO_{P10}$,

$$\alpha = \sum_{k=1}^{n} (DO_{obs} - DO_{P10})_k \in \mathrm{Z}^- \text{(in days)} \qquad (7a)$$

if $DO_{obs} > DO_{P90}$,

$$\beta = \sum_{k=1}^{n} (DO_{obs} - DO_{P90})_k \in \mathrm{Z}^+ \text{(in days)} \qquad (7b)$$

where DO_{obs} is the disease onset observed in experimental situation k (expressed in days since emergence), DO_{P10} and DO_{P90} are respectively the disease onset predicted at 10th and 90th percentiles of the *cDIV* distribution (expressed in days since emergence) and α and β quantify respectively the total prediction errors (in days) when disease onsets were either earlier or later than the predicted window. The value of τ that resulted in the lowest values of both α and β was chosen as the best RH threshold.

Statistical analyses

The effect of trap location (A, B, C or D) on weekly airborne inoculum availability was analysed with the GENMOD (generalized linear model) procedure of the SAS software package (SAS Institute Inc., Cary, NC) assuming a Poisson distribution of lesion counts and using the log link function. The CONTRAST statement was used to perform custom hypothesis tests such as the equality of the numbers of lesions per trap plant at upwind locations A and B.

The effect of mesoclimatic variables on airborne primary inoculum availability was investigated through multiple regressions by using the stepwise model-selection method of the REG procedure of SAS. The F statistic for a variable to be included and to stay in the model had to be significant at the 0.05 level. Simple residuals (predicted minus observed values) were tested for normality using the UNIVARIATE procedure (Shapiro-Wilk and Kolmogorov-Smirnov tests). The performance of the model was analysed using a regression approach (prediction versus observation). The estimated values of the slope and intercept of the fitted model ($y = ax + b$) were compared to those of the $y = x$ line (i.e., we tested whether the slope was equal to 1 and whether the intercept was equal to 0) using a t-test.

Results

Seasonal dynamics of airborne inoculum availability

Depending on field experiments, airborne inoculum availability was assessed indirectly using trap plants for 27 or 36 weeks (Fig. 3). For both experiments, the
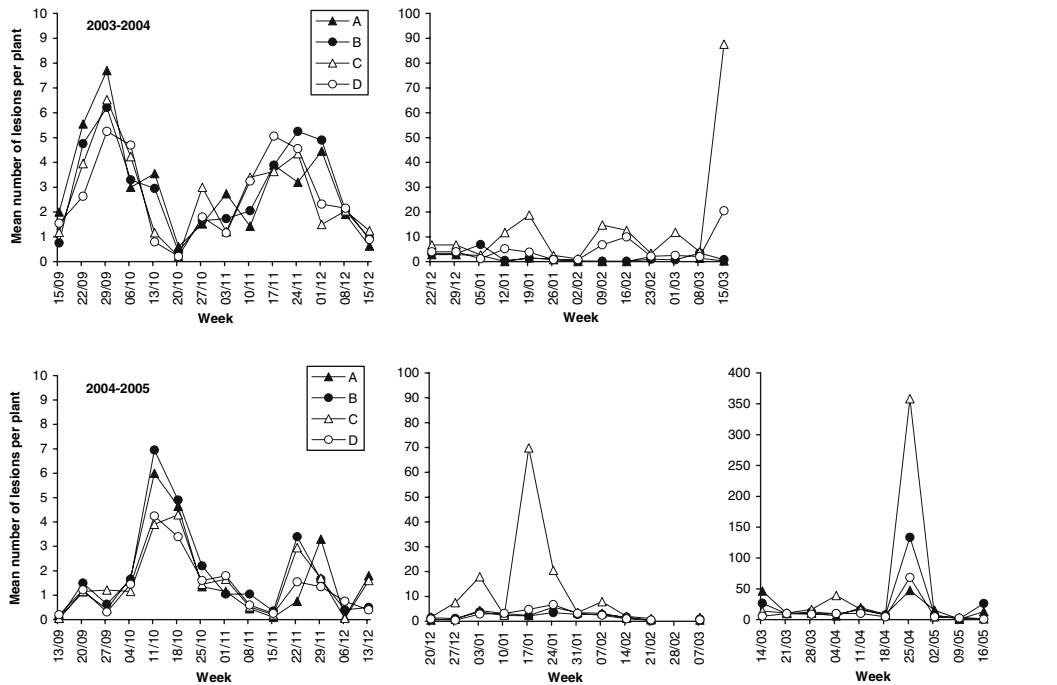
Fig. 3 Availability of aerial inoculum of ascochyta blight indirectly assessed on trap plants placed at the four corners of field experiments conducted in France in 2003–2004 and 2004–2005 cropping seasons. A and B are upwind trap plants. C and D are downwind trap plants

seasonal pattern showed two main phases. The first phase (mid-September to mid-December) corresponded to a period during which there were generally no significant differences between the four trap plant locations. During this phase, inoculum availability was extremely low and very few lesions (between 0 and 8 lesions per plant per week) were observed. The second phase (mid-December to early/mid-March) corresponded to a period during which downwind trap plants (mainly location C) trapped significantly more airborne inoculum than upwind trap plants (locations A and B). During this phase, up to 90 lesions per plant per week were observed on the downwind trap plants. Low levels of airborne inoculum continued to be observed on the upwind trap plants. The fact that the high levels of inoculum were all restricted to downwind trap plants suggested that the corresponding inoculum was probably a pycnidiospore-like secondary inoculum splashed at short distance from early-sown infected plants. In addition to these two phases, the 2004–2005 seasonal dynamic showed a third phase (mid-March to mid-May) during which high levels of airborne inoculum were detected on both the upwind and downwind trap

plants. Up to 350 lesions per plant per week were observed in mid-April. The fact that high levels of inoculum were detected on downwind but also on upwind trap plants tends to support the hypothesis that this trapped inoculum was an ascospore-like secondary inoculum dispersed by wind at long distance from infected plants.

Relationship between airborne primary inoculum availability and mesoclimatic variables

Here we focused solely on the phases of the airborne inoculum availability dynamics corresponding to airborne primary inoculum. The levels of airborne inoculum availability assessed on upwind trap plants (locations A and B) from mid-September to mid-March were averaged to generate a new dependent variable, which was analysed using multiple regression methods. Variables derived from temperature (minimum, maximum, mean, sum of degree-days) and rainfall (sum, maximum, number of rainy days) were considered as potential explanatory variables. The best model selected by the stepwise method involved degree-days and maximum rainfall:
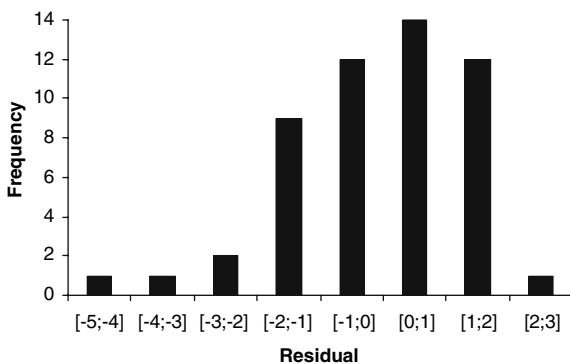
$$IP = 0.02198 \times DD + 0.11185 \times R_{max} (R^2 = 0.71)$$
$$(8a)$$

where IP is the level of primary inoculum expressed as lesions per plant and per week, DD is the sum of degree-days (from $0°C$) calculated during the corresponding week and R_{max} is the maximum daily rainfall measured during the corresponding week.
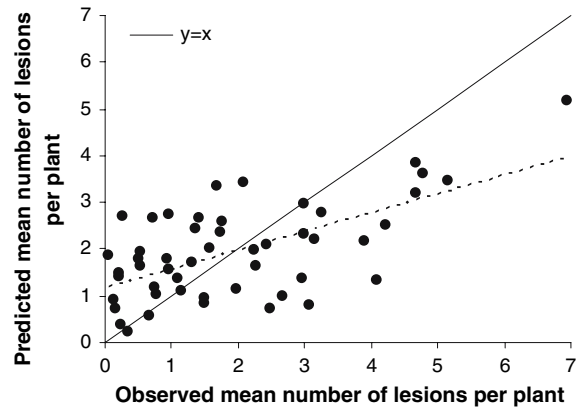
Simple residuals (predicted minus observed values) were normally distributed according to the Kolmogorov-Smirnov test ($P > 0.15$) and near-normally distributed according to the Shapiro-Wilk test ($P = 0.048$). The histogram of residuals showed that two under-estimated values (-3.96 and -4.40) had a great impact on the distribution (Fig. 4). Removing the corresponding observed values from the analysis improved the normality of the distribution (Shapiro-Wilk test: $P = 0.372$) and only affected the regression equation slightly:

$$IP^* = 0.0192 \times DD + 0.1171 \times R_{max} (R^2 = 0.76)$$
$$(8b)$$

However, the prediction of the weekly airborne primary inoculum availability given by this model has to be considered with caution. Indeed, the slope of the prediction versus observation regression line was significantly <1 (estimate = 0.395, standard error = 0.072, $n = 50$) and the intercept significantly >0 (estimate = 1.176, standard error = 0.183, $n = 50$) (Fig. 5).
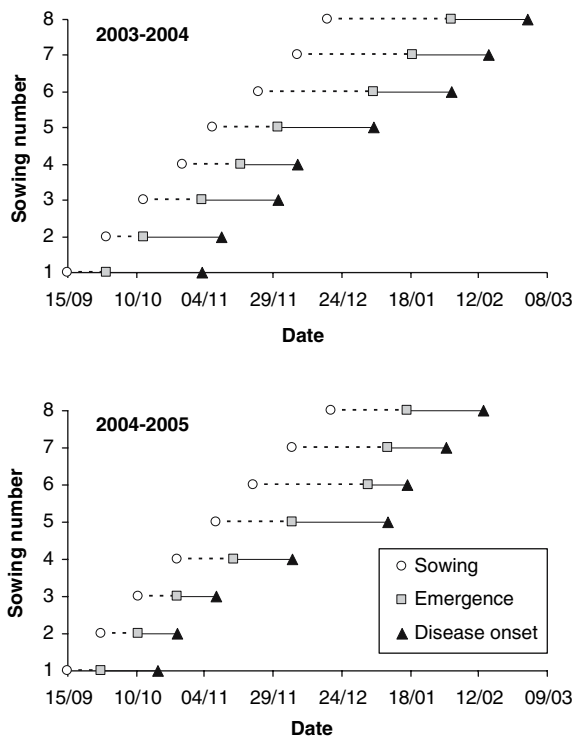

Fig. 5 Comparison between predicted and observed levels of airborne primary inoculum. Predicted values were calculated by the following equation: $IP^* = 0.0192 \times DD + 0.1171 \times R_{max}$ ($R^2 = 0.76$), where $IP*$ is the level of airborne primary inoculum expressed in lesions per plant and per week, DD is the sum of degree-days from $0°C$, calculated during the corresponding week and R_{max} is the maximum rainfall measured during the corresponding week. The dotted line represents the prediction versus observation regression line

Relationship between disease onset and sowing date

Disease onset occurred between mid-October and early March (Fig. 6). Generally, the later the sowing date, the longer the period between sowing and disease onset. This was mainly explained by an increase in the duration of the period between sowing and emergence. Disease onset was observed 14–35 days after emergence. Pea plants had 2–5 leaves when disease onset occurred. The length of the period between emergence and disease onset (expressed in days after emergence, DAE) appeared to be independent of sowing date. For instance, short periods were observed for either early or late sowing dates. In addition, regression analyses indicated that little variability in DAE was accounted for by mesoclimatic (such as cumulative degree-days or rainfall during the period) and biological (such as cumulative primary inoculum during the period) variables. Only a weak simple linear regression linking cumulative degree-days to DAE was found:

$$DAE = 0.066 \times DD + 11.898 \ (R^2 = 0.36) \qquad (9)$$

where DD is the cumulative degree-days (from $0°C$) between emergence and disease onset.

Fig. 4 Histogram of the simple residuals (predicted minus observed values) of the multiple regression established between airborne primary inoculum and mesoclimatic variables

Fig. 6 Schematic representation of sowing, emergence and ascochyta blight onset dates in field trials conducted in France in 2003–2004 and 2004–2005

This model suggested that cumulative degree-days could partially explain the variability of *DAE* in the sense that the greater the accumulation of degree-days, the later the disease onset. In fact, considering the known positive effect of temperature on infection processes, this was highly improbable. This relation simply illustrated the fact that with late disease onsets (i.e., more days between emergence and disease onset), cumulative degree-days are inevitably greater than with early onsets. This showed the limitations and pitfalls of this approach and suggested that rather than trying to explain disease onset with measured values of mesoclimatic variables, we should consider effective values (effective in the fungal infectious process) to predict the occurrence of disease onset.

Weather-dependent modelling of disease onset

The hypothesis underlying this approach is that disease onset occurs once the temperature and moisture requirements for incubation are met. Cumu-

lative daily infection values (*cDIVs*) varied between 0.5 and 11.2 according to the experiment, sowing occurrence and RH threshold (Table 2). As expected, the lower the RH threshold, the greater the *cDIVs*. For a 70% threshold, the 10th and 90th percentiles were 4.3 and 10.0, respectively. Using this threshold, observed disease onset dates were almost always included in the forecast window defined by the *P10* and *P90* predicted disease onset dates (Fig. 7). The observed date occurred before the forecast window in S8/2003–2004 (−4 days) and S6/2004–2005 (−2 days), and it occurred after in S1/2004–2005 (+1 day). Furthermore, the length of the predicted window varied greatly. Comparisons between the total prediction errors showed that the 70% RH threshold provided the best trade-off between the α and β type deviations (Fig. 8).

Discussion

Airborne primary inoculum availability

Airborne inoculum availability was assessed indirectly through trap plants. Trap plants, as opposed to Rotorod or Burkard spore samplers, are a robust way of assessing viable airborne inoculum. This method was well adapted for weekly assessment of inoculum release, and has been successfully used in previous studies concerning *M. pinodes* on pea (Roger and Tivoli 1996) or *Didymella rabiei* on chickpea (Trapero-Casas et al. 1996).

Under our experimental conditions, the amount of spores trapped in autumn and winter was extremely low and contrasted sharply with the amount of spores trapped in spring and attributed to ascospore-like secondary inoculum. How can the low amount of spores in autumn and winter be explained? Rainfall leaching of deposited spores is unlikely to be a significant factor, since according to the literature, ascospores of *M. pinodes* have a surface coating which causes them to adhere very firmly to objects with which they come into contact (Carter and Moller 1960). Thus, the low amount of spores trapped in autumn and winter may largely reflect a low level of airborne primary inoculum. This differs greatly from previous results concerning the seasonal release of ascospores of *M. pinodes* (Roger and Tivoli 1996; Zhang et al. 2005) or other ascomycota (Inman et al.

Table 2 Cumulative daily infection values (*cDIV*) calculated for various RH thresholds in field experiments conducted in France in 2003–2004 and 2004–2005

Year	Sowing number	RH threshold (%)						
		65	70	75	80	85	90	95
2003–2004	S1	nc	nc	nc	nc	nc	nc	nc
	S2	10.4	9.9	9.0	7.8	6.7	4.8	1.9
	S3	10.2	10.1	10.0	9.3	8.3	6.8	3.6
	S4	6.7	6.7	6.7	6.6	5.8	4.9	2.5
	S5	7.5	7.4	7.1	6.5	5.4	3.9	2.8
	S6	7.3	7.2	6.6	5.9	4.5	3.3	2.6
	S7	6.3	5.7	5.1	4.1	2.7	1.7	1.2
	S8	4.7	4.0	3.4	2.5	1.6	0.9	0.5
2004–2005	S1	11.2	10.6	9.7	8.6	7.5	5.6	3.7
	S2	8.2	7.9	7.3	6.1	5.1	3.9	2.7
	S3	6.0	5.8	5.0	4.5	3.8	3.0	2.1
	S4	5.7	5.6	5.4	5.2	4.5	3.4	2.4
	S5	7.8	7.7	7.4	7.1	6.3	5.0	3.0
	S6	3.7	3.6	3.5	3.4	3.3	2.9	1.8
	S7	4.8	4.7	4.6	4.4	3.9	3.2	2.1
	S8	6.3	6.2	5.8	5.4	4.6	3.1	1.7
	10th percentile	4.8	4.3	4.0	3.7	2.9	2.2	1.4
	90th percentile	10.3	10.0	9.4	8.3	7.2	5.3	3.4

In 2003–2004, the first disease assessment achieved on S1 revealed that disease onset had already occurred. Since disease onset was not accurately dated, the corresponding *cDIVs* were not calculated

1999; Salam et al. 2003; Trapero-Casas et al. 1996) probably because these authors placed their trapping systems (trap plants or spore samplers) directly in the centre or in the vicinity of infected debris. Our experiments were conducted in fields with a rotation that excluded pea during the previous five years. In addition, the nearest pea stubble was located, 1,600 m from the trial plots and was removed in early September before ploughing. It is therefore likely that the removal of the remaining debris would have drastically reduced the primary inoculum from this source.

In this study, airborne primary inoculum availability was partially explained by cumulative degree-days and maximum daily rainfall. Reports in the literature suggest that primary inoculum availability is highly dependent upon weather conditions. Under their experimental conditions, Trapero-Casas et al. (1996) observed that the number of lesions of *D. rabiei* on chickpea trap plants was significantly correlated with the number of days with rain during weekly periods. Zhang et al. (2005) showed that ascospores of *M. pinodes* were released 1–2 days after a rain event.

Modelling of disease onset

In this study, late sowing dates delayed disease onset. In Australia, this cultural practice is recommended to reduce the exposure of young plants to high levels of primary inoculum (Bretag et al. 2000). In French conditions, delaying sowing will mainly induce a delay in emergence due to decreasing temperatures during autumn and winter and consequently delay the deposition of the primary inoculum on young susceptible pea plants. Nevertheless, this practice also has limitations: firstly, delaying sowing can in some cases reduce yield (Bretag et al. 2000), and secondly, there is a risk that sowing may not be possible due to autumn rainfall.

Although assessed in very low quantities, primary inoculum appeared to be sufficient to initiate an epidemic. Carter and Moller (1960) reported that spores of *M. pinodes* were able to survive between conducive incubation periods (such as might be expected with overnight dews followed by dry days) and retain their ability to infect when favourable moisture conditions resume. Roger et al. (1999a) showed that symptoms were able to develop provided
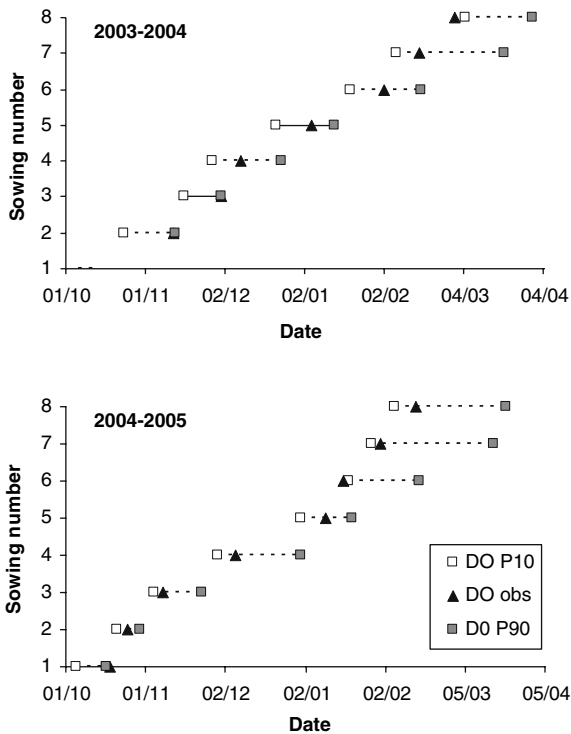
Fig. 7 Schematic representation of observed and predicted dates of ascochyta blight onset for field trials conducted in France in 2003–2004 and 2004–2005. DO_{obs} is the observed disease onset, DO_{P10} and DO_{P90} are respectively the predicted dates calculated for the 10th and 90th percentiles of the distribution of the cumulative disease infection values at 70% RH threshold
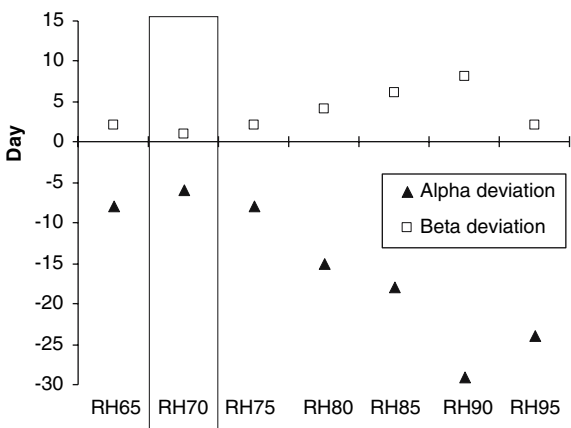
Fig. 8 Total deviations between observed and predicted ascochyta blight onset dates calculated for seven RH thresholds (65, 70, 75, 80, 85, 90 and 95%) from data obtained in field trials conducted in France in 2003–2004 and 2004–2005. Alpha and beta deviations are calculated respectively when observed disease onset occurred before and after the forecast window given by the model

that the dry period occurred after germination. Thus, the actual amount of ascospores available for disease onset in a given week is probably higher than the weekly availability assessed on trap plants and probably results not only from the weekly spore deposition but also from some viable spores from previous weeks.

Not much variability in the length of the time from emergence to disease onset was accounted for by mesoclimatic variables. A different modelling approach was used to predict the occurrence of disease onset. This was achieved through the calculation of cumulative temperature and moisture indices which were used to define a disease risk forecast window. A similar approach was used by Shane and Teng (1983) and Wolf and Verreet (2005) for *Cercospora beticola* and by Bugiani et al. (1993) for *Phytophthora infestans*. For ascochyta blight, we used data available in the literature to establish the mathematical functions. This study shows that the 70% RH threshold provided the best results for predicting disease onset. This rather low threshold (RH recorded at 1.40–2.20 m above ground level) is probably associated with optimal moisture periods within the crop canopy due to dew formation.

Wolf and Verreet (2005) proposed a negative prognosis (determining a disease-free period) based on the minimum value of the *cDIV* distribution. In our study, we chose to define the bounds of a forecast window in which disease onset is likely to happen. Except for three cases, the predicted forecast windows included the observed disease onset dates. For these three cases, the deviation varied between −4 and +1 (days), which is acceptable. The length of the forecast window was variable. The more conducive the weather conditions, the shorter the forecast window. Actual monitoring of the plants is therefore needed once the forecast window is reached and is all the more urgent if subsequent weather conditions are conducive.

To our knowledge, this study is the first attempt to predict ascochyta blight onset in field peas. Once validated with additional data, the basis of this model could be used to design a user-friendly tool to warn farmers about disease risk and possibly to advise them on an appropriate time for the first fungicide application. Therefore, this model could contribute to development of a more rational fungicide application schedule. Of course, such a tactical tool would not be of interest if fungicide applications are not econom-

ically beneficial. Finally, this model needs to be coupled to a disease progress model to predict the subsequent development of ascochyta blight and associated pea yield losses.

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REVIEW

Integrated disease management of ascochyta blight in pulse crops

Jennifer Anne Davidson · Rohan B. E. Kimber

Abstract Ascochyta blight causes significant yield loss in pulse crops worldwide. Integrated disease management is essential to take advantage of cultivars with partial resistance to this disease. The most effective practices, established by decades of research, use a combination of disease-free seed, destruction or avoidance of inoculum sources, manipulation of sowing dates, seed and foliar fungicides, and cultivars with improved resistance. An understanding of the pathosystems and the inter-relationship between host, pathogen and the environment is essential to be able to make correct decisions for disease control without compromising the agronomic or economic ideal. For individual pathosystems, some components of the integrated management principles may need to be given greater consideration than others. For instance, destruction of infested residue may be incompatible with no or minimum tillage practices, or rotation intervals may need to be extended in environments that slow the speed of residue decomposition. For ascochyta-susceptible chickpeas the use of disease-free seed, or seed treatments, is crucial as seed-borne infection is highly effective as primary inoculum and epidemics develop rapidly from foci in favourable conditions. Implemented fungicide strategies differ according to cultivar resistance and the control efficacy of fungicides, and the effectiveness of genetic resistance varies according to seasonal conditions. Studies are being undertaken to develop advanced decision support tools to assist growers in making more informed decisions regarding fungicide and agronomic practices for disease control.

Introduction

Ascochyta blight is the most severe foliar disease of cool season pulses, the major crops being chickpea, faba bean, lentil and field pea, and severe epidemics may result in total crop failure. Pathogens that cause ascochyta blight belong to *Ascomycota;* they have worldwide distribution and are predominantly host-specific. *Ascochyta rabiei* (teleomorph: *Didymella rabiei*), *Ascochyta lentis* (syn. *A. fabae* sp. *lentis*) and *Ascochyta fabae* (teleomorph: *Didymella fabae*) infect chickpea, lentil and faba bean, respectively. Ascochyta blight of field pea is caused by a complex of three fungal species; *Ascochyta pinodes* (teleomorph: *Mycosphaerella pinodes*), *Ascochyta pisi* and *Phoma medicaginis* var. *pinodella*, formerly known as *Ascochyta pinodella*. This highly efficient group of pathogens undergo heterothallic sexual reproduction

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on infested residue, resulting in air-borne ascospores, which are capable of spread over long distances. Rapid polycyclic spread within crops occurs over short distances through splash-borne asexual conidia (pycnidiospores). The disease affects all above ground parts of the plant and is characterised by necrotic lesions, which on susceptible cultivars in favourable conditions, can girdle stems leading to breakage and severe yield reduction. Seed quality may also be reduced through seed discolouration or retardation of seed development.

Significant improvements in host resistance are being realised in breeding programmes and a greater understanding of integrated disease management options can reduce the incidence, severity or persistence of ascochyta blight. Nevertheless, control of this aggressive disease continues to challenge pulse industries and researchers worldwide, and ascochyta blight epidemics continue to depress yields.

This review summarises the current knowledge of the management of ascochyta blight in the pulse crop. Managing ascochyta blight firstly relies on minimising the onset of disease epidemics by reducing or avoiding primary inoculum, and secondly by suppressing the subsequent epidemic increase using resistance or foliar fungicides. Methods of control include destroying or avoiding inoculum sources, crop rotations, manipulating sowing times, sowing disease-free seed, applying seed and foliar fungicides and adopting cultivars with improved resistance. The combination of strategies is determined by economics, availability of cultivar resistance and disease epidemiology.

Avoiding infested residue and in situ inoculum

Ascochyta fungi survive on infested crop residue lying on the soil surface and for a reduced period of time on buried residue. Asexual reproduction on residue gives rise to pycnidia, which exude pycnidiospores, spread via rain splash, whereas sexual reproduction forms pseudothecia, which discharge ascospores capable of spread over long distances by wind and rain. *Mycosphaerella pinodes* and *P. medicaginis* var. *pinodella* can also produce chlamydospores, long-term soil-borne survival structures that may persist for at least 5 years (Wallen and Jeun 1968), and pea crops become infected if they are planted in soils containing this soil-borne inoculum.

The management of infested residue and soils is an important component of controlling ascochyta blight. Where ascospores are the major source of infection, crop rotation is less effective, and crop isolation and residue burial will be more beneficial.

Proximity to infested residues

Isolation from infested residue is an important strategy in all cool-season pulse crops to avoid ascochyta diseases. Ascospores are wind-dispersed and may spread long distances: at least 400 m in the case of *M. pinodes* (Davidson et al. 2006; Galloway and MacLeod 2003) and 100 m in the case of *A. rabiei* (Trapero-Casas et al. 1996) though the distances may be greater if spores are blown in air currents (Kaiser 1992). In addition to ascospores, infested residue may be blown into neighbouring crops. In Australia, crop residues are considered the most important source of ascochyta inoculum for faba bean (Hawthorne et al. 2004) and field pea crops (Bretag et al. 2006; Carter and Moller 1961). Disease gradients across crops clearly indicated that wind-blown spores or infested debris from neighbouring crop residue acted as primary inoculum in lentil crops in Canada (Morrall 1997) and bean crops in the UK (Bond and Pope 1980). In the latter study, a decreasing frequency of ascochyta blight on beans, from the border to the centre of the field, for a distance of 120–200 m, suggested that spread from adjacent fields was more important than seed infection, whereas previously most outbreaks in the UK and Canada had been attributed to seed infection (Hewett 1973).

Burial of infested residue

Burial of debris hastens residue and pathogen decomposition thereby reducing the inoculum loads. *Ascochyta rabiei* inoculum on buried chickpea residue is no longer viable after 2–5 months. In contrast, inoculum is still viable on residue on the soil surface after 2 years (Gossen 2001; Kaiser 1973; Navas-Cortes et al. 1995; Nene and Reddy 1987). Zhang et al. (2005) found that *M. pinodes* spore production from buried pea residues rarely continued after 11 months regardless of depth of burial, but higher numbers were produced on residues on the soil surface. Similar results were found in Australia

(Davidson et al. 1999). Decomposition is aided by environments of high temperatures and adequate moisture but in extreme environments of less than −40°C or more than +40°C, such as in Canada, residue breakdown is inhibited. In studies examining survival of *A. rabiei* (Gossen and Miller 2004) and *A. lentis* (Gossen 2001) on infested residues, the pathogens were able to survive when buried within the soil profile for more than 4 years, albeit at a low level of pathogen recovery. Hence two or even three non-host crops are needed between successive chickpea or lentil crops to reduce the risk of an epidemic developing (Gossen and Derksen 2003; Gossen and Miller 2004). However, in the Pacific Northwest of USA the pathogens survive for a shorter period of 3 years on buried residue (Kaiser and Hannan 1986). These differences indicate that regional environments influence the speed of residue decomposition, rather than directly impacting on pathogen survival. Nevertheless, burying residue reduces the spread of pycnidiospores and ascospores by preventing exposure for splash or wind dispersal.

Burying residue may reduce spore production and hasten decomposition but it is incompatible with no or minimum tillage practices. In addition, even after several passes with tillage equipment, some residue remains on the soil surface (Gossen and Miller 2004). Burning residue is another tool to effectively destroy inoculum but has also become less popular in many regions due to environmental concerns. The increasing amount of plant residue left on the soil surface with minimum tillage is thought to be a potential hazard for increasing the severity of epidemics, and alternative means of suppressing the pathogens are required. Studies are underway to investigate the potential of using biological control to suppress *A. rabiei* on chickpea residue, concentrating on fungal colonisers such as *Aureobasidium pullulans* and *Clononstachys rosea* (Dugan et al. 2005).

Soil borne inoculum and crop rotation

The recommended interval between like pulse crops to minimise ascochyta infection is governed by the speed of residue breakdown. Crop rotation between 3 and 6 years is recommended in most regions to avoid *in situ* inoculum, while in warm, moist areas of the world, rotations of 1 or 2 years with a non-host is sufficient (Kaiser et al. 2000). The pathogens may survive directly on the residue of previous crops, which in many environments will decompose much quicker if buried.

However, the causal pathogens of ascochyta blight on field pea (*M. pinodes* and *Phoma medicaginis* var. *pinodella*) can survive in soil as mycelium or chlamydospores (Hare and Walker 1944; Wallen and Jeun 1968) and *M. pinodes* is a moderately successful saprophyte (Dickinson and Sheridan 1968). The longevity of these structures influences the period that is required between pea crops. In Australian farming systems, rotation interval between pea crops has recently increased from 3 to 5 years, to avoid infection from in situ inoculum. Disease severity was greater in crops sown on shorter rotations compared to those on longer rotations and yield, based on grower data, was consistently lower in the shorter rotation crops (Davidson and Ramsey 2000). Bretag et al. (2001) monitored changes in populations of soil-borne ascochyta blight fungi, following different cropping sequences of field pea and barley. Inoculum levels were twelve times higher following 3 years of field pea compared to 3 years of barley. Yield losses of field pea sown in the fourth year were highly correlated to the level of soil borne fungi. Similar studies in the USA found that *P. medicaginis* var. *pinodella* could be isolated from soil that had not been sown to pea for up to 5 years, while *M. pinodes* was isolated from soils that had not grown pea for over 20 years (Wallen and Jeun 1968). These results bring into question the effectiveness of a three-year rotation between pea crops to reduce ascochyta blight. Davidson et al. (2001) investigated survival of ascochyta blight pathogens in soils of commercial pea-cropping paddocks. While soil populations were found to degrade over time, the pathogen population levels varied widely between paddocks with the same paddock history. Hence relying on a simple paddock rotation may not be sufficient since crops could be planted in soils with potentially damaging levels of pathogens. It is likely that the level of pathogen populations in the soil is related to the severity of the epidemic in the last pea crop grown.

Studies on the survival of *A. fabae*, from soil samples taken to a depth of 5 cm, concluded that this pathogen does not survive for even a few months directly in field soil (Wallen and Galway 1977). This is probably due to the inability of the pathogen to

form chlamydospores, making it dependent upon the presence of infested residue for survival. While viable inoculum remains on infested residue in the field, rotations are still a primary means of disease control in faba bean and a three-year rotation is recommended in Australian conditions (Hawthorne et al. 2004). Residue is also regarded as an important source of inoculum for *A. fabae* in Iraq (Michail et al. 1983).

Sowing date

Ascospores are released into the air from infested residue at certain times of the year, depending on environmental conditions, and sowing date of crops can be manipulated to avoid the maximum risk period when airborne ascospore are at their highest numbers.

In Australia, pea crops are sown two to three weeks after the agronomic optimum to avoid the peak period of ascospore release which occurs at the beginning of the growing season (Bretag 1991). Earlier sown crops have the most ascochyta and the highest percentage of infected grain at harvest (Bretag et al. 2000) particularly in the most intense pea cropping areas (Davidson and Ramsey 2000). In higher rainfall areas later planting has less impact on yield (Davidson and Ramsey 2000), but this practice risks yield loss in short growing seasons and regions where spring rain is limiting, with losses as high as 40% in some later-sown crops (Bretag et al. 2000).

This situation also occurs in chickpea where the maximum ascospore numbers may coincide with emergence of chickpea crops (Trapero-Casas et al. 1996). In southern Spain a delay in sowing date reduces the disease risk to emerging crops from airborne ascospores. However, as with field pea, delayed sowing can adversely affect yield if it compromises the optimum agronomic sowing date (Gan et al. 2005).

Where ascospores are not the primary source of inoculum, or ascospore release does not coincide with sowing date, delayed sowing of susceptible cultivars of chickpea and lentil is still often recommended to reduce the window of protection required by fungicides to keep ascochyta under control (Gan et al. 2005; Materne et al. 2001). Due to the polycyclic nature of ascochyta, later sowing lowers the epidemic intensity by limiting the number of pycnidiospore cycles.

In some situations, the main source of inoculum may be produced on early-sown crops, providing inoculum for later-sown crops, which can then become severely affected. Late-sown pea crops in some regions of the Northern Hemisphere suffer more ascochyta for this reason (Hare and Walker 1944).

Diseased seed and fungicide seed treatment

Diseased seed

Infected seed is a means of introducing ascochyta blight to new areas and there are numerous reports of ascochyta blight pathogens being introduced via infected seed (Ali et al. 1982; Bretag et al. 1995; Cother 1977a, b; Galdames and Mera 2003; Gossen and Morrall 1986; Kaiser 1997; Kaiser and Hannan 1986; Kaiser and Muehlbauer 1984; Morrall and McKenzie 1974). The proportion of seeds infected with *A. rabiei* in tested chickpea samples has been recorded as high as 70% in Turkey (Maden et al. 1975), while in the Pacific Northwest, USA, infection of commercial seed lots varied from 0.5 to 31% (Kaiser 1992). High levels of *A. lentis* infection have also been recorded in lentil seed lots, with 20% infection detected in Ethiopian seed lots (Ahmed and Beniwal 1991). Seed testing is a major component of *A. lentis* control in Canada (Morrall 1997) and Australia (Lindbeck et al. 2002). The importance of this was particularly demonstrated in the latter country where 33% of seed lots tested across the nation were infected, with higher incidence on seed harvested from earliest sown crops (Nasir and Bretag 1997). The importance of seed infection as an inoculum source is dependent on several factors; % of seed infection, the rate of seed to seedling transmission, the developmental rate of an epidemic from seedling foci, and the comparative influence of alternative sources of inoculum.

Seed to seedling transmission

Seed transmission rates for *A. rabiei* have been reported as 5% in field conditions (Kimber et al. 2007) to 20–30% in glasshouse conditions (Kimber et al. 2006; Maden 1983). The production of disease-free seed is seen as an important strategy in Pakistan,

in areas free from *A. rabiei* infection (Mitsueda et al. 1997). The use of disease-free seed is crucial for susceptible chickpea cultivars as seedling foci rapidly develop into epidemics in conditions conducive to disease development (Kimber et al. 2007). The rapid spread of ascochyta blight from primary infections in susceptible chickpea cultivars led to the development of a more rigorous PCR-based seed test in Australia. The original seed test (400 seeds on culture medium) was based on a procedure recommended by the International Seed Testing Association (ISTA 1996) and was able to detect as low as 0.25% infection levels. However, even lower levels of infected seed (0.01–0.1%) are sufficient to initiate epidemics when weather conditions are favourable (Kaiser 1992; Kimber et al. 2007). The PCR test uses DNA primers specific to *A. rabiei*, based on sequencing of the internal transcribed spacer region of the ribosomal gene complex. This test can detect DNA from 10 spores in a PCR reaction (Ophel-Keller et al. 1999). Comparisons between the PCR test, which uses 1,000 seed samples, and the plating test, were conducted on 50 seed lots. The PCR test was positive in all 13 cases where the plating test was positive, but it also detected a further 10 cases of *A. rabiei* infection in samples not detected by the plating technique. Some of these 10 cases were associated with severe ascochyta epidemics, where PCR testing was conducted post-sowing (Ophel-Keller et al. 1999), emphasising the need for the more sensitive procedure. Testing revealed that the majority of seed lots in Australia were infected and, in the absence of locally adapted resistant cultivars, the industry rapidly declined in regions conducive to ascochyta epidemics.

The majority of research on ascochyta control in lentil has concentrated on seed treatments and resistant cultivars. In Canada and Australia, stringent seed standards are recommended for lentil. Seed transmission rates in this crop appear to be low (Ahmed and Beniwal 1998) especially in dry soils of more than 15°C, but higher in wet soils at 8°C (Gossen and Morrall 1986). Western Canadian farmers plant ascochyta-infected lentils but levels below 5% seldom cause epidemics (Morrall 1992; Morrall and Sheppard 1981); however in areas of higher rainfall that promote epidemics, pathogen-free seed should be used (Morrall and Bedi 1990).

Infected seed is considered a major source of inoculum for *A. fabae* in the UK (Hewett 1973), Iraq (Michail et al. 1983) and New Zealand (Gaunt and Liew 1981). Transmission rate was estimated at 1–3% in Canada (Wallen and Galway 1977), and 4–8% in the UK (Hewett 1973). In the latter country, seed with more than 3% infection is discarded, and 1–3% infection is treated with a seed dressing (Jellis et al. 1998). There are varying reports on the importance of *A. fabae* seed infection in western Canada. Wallen and Galway (1977) found that after sowing seed with 13% infection, only 1% of harvested seed was infected. However, other studies in western Canada found that 1–5% seed infection could result in 27–35% infection on harvested seed (Bernier 1980; Kharbanda and Bernier 1979). Differences are likely to be due to environmental seasonal effects. In New Zealand, a significant yield reduction of 44% was observed due to disease that developed from seed with 12% initial infection. Infected seed affects plant establishment and disease incidence (Gaunt and Liew 1981). Control strategies were recommended for seed production crops including seed testing, a seed treatment of benomyl and captan, followed by a foliar spray of chlorothalonil during podding to prevent seed infection (Gaunt and Liew 1981; Hampton 1980).

Several studies have found no correlation between the level of *M. pinodes* seed infection in field pea and the severity of ascochyta on subsequent foliage (Bretag et al. 1995; Moussart et al. 1998; Xue et al. 1996; Xue 2000). Moussart et al. (1998) concluded that while *M. pinodes* seed infection resulted in disease at the basal parts of the plant as a foot rot symptom, no aerial symptoms were seen and so seed was not regarded as a source of contamination in the epidemiology of the disease. Xue (2000) found a high seed to seedling transmission of *M. pinodes* (70–100%), also leading to foot rot as well as reduced emergence, yield and seed weight. Seed to seedling transmission rate varies depending on environmental conditions (Bretag et al. 1995; Xue 2000) in that in drier regions transmission is of minor concern (Bretag et al. 1995). Seed infection levels >10% significantly reduce emergence (Bretag et al. 1995; Wallen et al. 1967; Xue 2000) but a higher seeding rate can compensate for this loss (Bretag et al. 1995). However, seed infection is important in areas where pea is seldom grown since it introduces the disease to

new areas. Seed infection can be reduced by avoiding seed lots produced from crops with high levels of ascochyta blight, such as early-sown crops, and avoiding late-harvested crops in which the disease has had more time to develop and infect seeds (Bretag et al. 1995).

Fungicidal seed treatments

Seed treatments reduce but do not completely inhibit the transfer of the pathogen to seedlings (Bernier 1980; Kaiser and Hannan 1987, 1988; Demirci et al. 2003). Nevertheless these treatments play an important role in reducing disease, particularly when combined with seed testing to minimise early establishment of the pathogens. Benomyl, carbendazim, chlorothalonil, thiabendazole, thiram and mixtures of these were effective in reducing seed to seedling transmission in pulse crops (Ahmed and Beniwal 1998; Grewal 1982; Kaiser et al. 1973; Kaiser and Hannan 1988; Kimber and Ramsey 2001; Reddy and Kababeh 1984; Rahat et al. 1993). Seed treatments are particularly beneficial for ascochyta control on chickpea and lentil.

Gan et al. (2005) summarised the physical and chemical methods that have been used to treat chickpea seed for *A. rabiei* infection. Excellent control, whereby the fungus was eradicated in laboratory tests and reduced infection to a minimum in field trials, was achieved using benomyl plus thiram, maneb, thiabendazole, or tridemorph plus maneb. Kaiser and Hannan (1988) and Maden (1983) found that benomyl and thiabendazole were the most effective of the fungicides tested and reduced seed infection on *A. rabiei* from 45% incidence to 0%. In laboratory conditions, thiram plus thiabendazole and carboxim plus thiabendazole reduced seed infection from an initial 80% to less than 5% (Kimber and Ramsey 2001).

Thiabendazole and carbendazim or benomyl have proven to be effective seed treatments on lentil (Bretag 1989; Kaiser and Hannan 1987). Iqbal et al. (1992) found that a range of tested fungicides reduced the recovery of seedborne *A. lentis* but most efficacious were Calixin-M, Benlate and Topsin-M. Lentil seeds with 81% infection had greater emergence when treated with thiabendazole or benomyl and yield was highest in thiabendazole-treated seeds (Kaiser and Hannan 1987).

A number of studies found that the benefits of using seed treatments to control ascochyta infection on faba bean and field pea are inconclusive, possibly because airborne inoculum has a greater influence on the ascochyta diseases on these crops, than does seed-borne inoculum. While a range of fungicides are effective at reducing seed-borne inoculum in laboratory assays on both field pea and faba bean (Karbanda and Bernier 1979; Wallen et al. 1967) the treatments have not shown a consistent reduction in plant infection (Karbanda and Bernier 1979; Michail et al. 1983; Thomas et al. 1989; Wallen et al. 1967) or any difference in emergence, disease severity or yield (Walsh et al. 1989). Thiram can increase emergence of infected field pea (Xue 2000) and a mixture of thiram and thiabendazole reduced ascochyta levels in pea at early growth stages (Davidson, unpublished data), but by flowering there was no effective disease control or yield gain at harvest (Davidson, unpublished data; Xue 2000). Conversely, Bretag (1985) demonstrated a small yield gain associated with thiabendazole seed treatment on field pea brought about by reducing the severity of ascochyta blight. Thiabendazole seed treatment is recommended on faba beans but alone does not provide sufficient protection (Jellis et al. 1998).

Foliar fungicides

A range of broad-spectrum foliar fungicides has been tested against ascochyta blight with varying results e.g. Bordeaux mixture, captan, captafol, chlorothalonil, folpet, mancozeb, maneb, metiram, wettable sulphur, zineb (Nene 1982; Sadkovskaya 1970; Warkentin et al. 1996, 2000). These are used as preventative sprays, and need to be applied before disease becomes established, or before rain events during which new infections occur. Chlorothalonil is the most widely used fungicide in ascochyta control and is the most consistent performer in reducing ascochyta blight on pulses (Ahmed and Beniwal 1991; Chongo et al. 2003; Gan et al. 2005; Kimber and Ramsey 2001; McMurray et al. 2006; Shtienberg et al. 2006). For faba bean, lentil and partially-resistant chickpea cultivars, foliar sprays of chlorothalonil are generally effective when applied at early flowering to early pod set (Kharbanda and Bernier 1979; Beauchamp et al. 1986a, b; McGrane et al.

1989; Ahmed and Beniwal 1991). At lower rainfall sites, a single spray during podding may be sufficient to protect against yield loss, reflecting the importance of the environment on epidemiology and disease spread (Beauchamp et al. 1986b). In Australian lentil crops, chlorothalonil or mancozeb are recommended during podding only if the disease is present and conditions are conducive to infection (Lindbeck et al. 2002). Chlorothalonil is applied to faba bean six weeks after sowing in Australia (Hawthorne et al. 2004) to protect against ascospore showers released from neighbouring infested residue (Galloway and MacLeod 2003). Follow-up sprays are applied during flowering and podding if disease is evident and conditions are conducive to disease (Hawthorne et al. 2004). The poor economics of foliar fungicides on field pea usually excludes this practice from commercial cropping.

Some systemic fungicides are also effective e.g. azoxystrobin, benomyl, carbendazim, thiabendazole and tebuconazole (Chongo et al. 2003; Demirci et al. 2003; Shtienberg et al. 2000; Thomas and Sweet 1989; Warkentin et al. 1996). These have the added advantage that they may be applied post-infection, or post rain event, though such applications may have the added complexity of conditions being unsuitable for ground-rig equipment. These fungicides penetrate the host tissue and possess post-infection properties, which enable them to be applied in the three days after infection has occurred (Shtienberg et al. 2000). Application of systemic fungicides post-infection allows for flexibility in management and reduces fungicide applications to real infection events rather than forecast events as with protective fungicides.

The disease pressure, environmental conditions and coverage achieved by the application, influence the efficacy of foliar fungicides. Foliar fungicides used on susceptible chickpea cultivars in many parts of the world (summarised in Gan et al. 2005) show that even with multiple applications, ascochyta might not be controlled under epidemic situations (Reddy and Singh 1992; Shtienberg et al. 2000). In Canada and Australia, in the presence of *A. rabiei*, the production of chickpea is rarely successful when highly susceptible cultivars are grown, despite multiple fungicide applications (Bretag et al. 2003; Chongo et al. 2003; Kimber and Ramsey 2001). Even under moderate disease pressure, four to six sprays became necessary to significantly reduce

disease. Only under dry conditions could fungicide applications be reduced (Chongo et al. 2003). In susceptible chickpea cultivars fungicides are generally uneconomic and impractical (Nene and Reddy 1987) and the rate of disease spread makes it difficult to follow an application schedule.

Preventative sprays are more effective when applied ahead of rain events during which infection occurs. The efficacy of chlorothalonil and mancozeb in Australian chickpea fungicide trials was reduced when the fungicide was not applied in time to protect crops from a rain event (Shtienberg et al. 2006). Analysis of the time of spraying in relation to rain events identified that disease was suppressed when fungicides were applied in time to protect plants from infection, but if plants were not protected during rain events, then control efficacy was low. The coincidence between control efficacy and uncontrolled rain was high i.e. $P < 0.01$, $R^2 = 0.937$ (Shtienberg et al. 2006). Management practices take this into account by encouraging continuous sprays of chlorothalonil every three weeks during the growing season. Simulated analysis of the trial data indicated that rain forecasting, to time fungicide sprays with rain fronts, could reduce the number of applications needed to control the epidemic. Initiating sprays after the presence of disease was confirmed, further reduced the number of sprays required for effective disease control.

Foliar fungicides on field pea have generally been uneconomic despite the reduction in disease and associated yield increases. Highly susceptible cultivars responded more to the fungicides than moderately susceptible cultivars (Warkentin et al. 2000), but even in these crops little spraying of field pea is conducted since multiple applications may be required to achieve significant disease suppression. Multiple sprays, initiated at early to mid-flowering provided some disease control and yield gains (Warkentin et al. 1996; Warkentin et al. 2000). A single application of mancozeb or chlorothalonil at early flowering also increased yields while a single late flowering application generally had no impact (Warkentin et al. 2000). Fungicide trials were conducted in Australia (Davidson, unpublished data) using mancozeb at 6, 9 and 12 weeks after sowing. Neither mancozeb nor chlorothalonil effectively controlled the disease and there were no yield gains in these trials. As breeding programmes develop

higher yielding cultivars, or the economic returns for pea increase, the financial benefit of applying foliar fungicides to field pea may also improve.

Strategic application of fungicides taking into account host resistance

Ascochyta resistance is a major priority in pulse breeding programmes around the world. No cultivars from these programmes have complete resistance, or immunity, to ascochyta due to the complexity of the host–pathogen relationship, but a number of cultivars exhibit partial resistance.

Ascochyta-susceptible pulse cultivars have been reliant on foliar fungicides but integrating enhanced resistance combined with clean seed and wide rotations has reduced foliar sprays and enabled the use of earlier sowing dates to maximise yield. Furthermore the lower input costs associated with reduced fungicide usage has greatly improved the economics of growing these crops. In Australia, foliar fungicides for ascochyta control in lentil crops are applied only at the podding stage since most Australian cultivars have foliar resistance to this disease (Lindbeck et al. 2002).

Partial resistance in chickpea is essential for the success of this crop in many parts of the world though the resistance can still be overcome in regions that have moderate to high inoculum pressure and weather conditions favourable to epidemics (Chongo et al. 2003). Two to four applications of chlorothalonil or azoxystrobin at early and mid-flowering are required under high disease pressure on partially resistant cultivars (Bernier 1980, Chongo et al. 2003; Kharbanda and Bernier 1979; Reddy and Singh 1992). In dry seasons a single spray on a moderately resistant cultivar may be sufficient (Pande et al. 2005). In some cases fungicide applications during podding are maintained to prevent pod infection, seed abortion or seed infection (Hawthorne et al. 2004) since resistance in chickpea is not as effective at flowering and podding (Chongo and Gossen 2001; Singh and Reddy 1993). Fungicide strategies differ according to cultivar resistance (Shtienberg et al. 2000) and the control efficacy of fungicides and effectiveness of genetic resistance vary according to seasonal conditions. When environmental conditions support severe epidemics, foliar fungicides may provide <20% control efficacy on susceptible and moderately susceptible cultivars, but as much as 70% control efficacy on moderately resistant cultivars. In mild epidemics >80% control efficacy is achieved on susceptible cultivars, and >95% on moderately susceptible and moderately resistant cultivars (Shtienberg et al. 2000).

Decision support systems

An understanding of the pathosystems and the interrelationship between host, pathogen and the environment is essential to be able to make correct fungicide and agronomic decisions for disease control. Some studies have been undertaken to develop decision support tools to assist growers in making these decisions.

Jhorar et al. (1997) studied weather data over a 27-year period in association with ascochyta blight of chickpea. Weekly averages of temperature, relative humidity (RH), sunshine duration, and total weekly rainfall and raindays were calculated for the period of vegetative growth to maturity. Disease at time of maturity was correlated with each of these parameters. A ratio of afternoon RH and maximum temperature was calculated to produce a parameter termed the humid thermal ratio and this was highly correlated with disease, $R^2 = 0.90$. This parameter was suggested as a useful model for disease prediction for fungicide applications.

In Israel, a predictive model determined that pseudothecial maturation and ascospore discharge of *A. rabiei* occurs after six rain events of equal to or >10 mm (Shtienberg et al. 2005). Fungicide applications at this time target the primary inoculum of ascospores and should prevent the infection of new crops and possibly the necessity of further fungicide applications in the crop. Subsequent sprays are initiated by monitoring, beginning when ascochyta is first observed in the crop, and are linked to forecasted rain thresholds for different cultivar resistances i.e. 5 mm for highly susceptible cultivars, 10 mm for moderately susceptible, 20 mm for moderately resistant, and 50 mm for resistant cultivars (Shtienberg et al. 2000).

A modelling system for ascochyta in field pea was developed by Salam et al. (2006) to predict time of onset, and progression of ascospore maturity and

spread of spores from the source of infection of *M. pinodes*. This model incorporates effects of rain, temperature and wind on fungal maturation, spore release and spore dispersal. The model Blackspot Manager helps growers to make decisions on when to sow their crop, by using year to date weather data and forward projection of historical data, to predict the likely ascospore load at a particular time of the year. The model also assists growers to select the best fields for field pea location to minimise the risk of ascochyta blight for several years in advance.

In the absence of effective resistance or economic fungicides, agronomic measures must be used to make decisions for ascochyta control in field pea. Multiple regression analysis of disease severity, cropping practices (i.e. sowing date, pea rotation history, proximity to infested residue) and environmental data, including cumulative rainfall and mean temperature, were used to predict ascochyta blight severity in field pea in South Australian cropping systems (Schoeny et al. 2003). The model is aimed at assisting growers to make informed decisions regarding rotations of pea crops and sowing date to minimise ascochyta.

Conclusion

Management of ascochyta is an essential component of successfully growing pulse crops. Where possible, moderately resistant cultivars should be grown but growers will select cultivars depending on yield, seed quality and marketability, not just on ascochyta resistance. Hence cultivars with different levels of ascochyta resistance will be grown and must be managed accordingly.

Integrated disease management includes a combination of cultivar resistance, seed and crop hygiene, seed and foliar fungicides and appropriate sowing dates. Selecting the most effective strategies can be difficult due to the complexity of the pathosystems and the inter-relationship with resistance and the environment. Decision support tools are in their infancy and rely on a good understanding of the epidemiology of the pathogens and the influence of the environment on the development and spread of the disease. As more research is conducted these tools will become more specific to crops, diseases and regions, enabling a good understanding of the forces that drive an epidemic. The challenge will then be to translate this information into a form that is understandable and useable by the grower to make agronomic and disease management decisions that are cost-effective and beneficial to yield and finances.

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REVIEW

The sympatric Ascochyta pathosystems of Near Eastern legumes, a key for better understanding of pathogen biology

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Abstract The primary and secondary centres of origin of domesticated plants are often also the places of origin of their pathogens. Therefore, the Near Eastern cradle of agriculture, where crop plants, their wild progenitors, and other congeneric taxa grow sympatrically, may hold some clues on the biology of the pathogens of the respective crops. Unlike the situation in the well-studied Near Eastern cereals and their important diseases, hardly any data are available on basic questions regarding grain legumes. What is the role of genetic diversity at resistance loci of the wild hosts and is it greater compared with the cultigens? Are populations of Ascochyta pathogens infecting wild legumes genetically distinct from populations infecting their domesticated counterparts, and if so, is this differentiation related to differences in host specialization or to adaptation to different ecological conditions? Do isolates originating from wild taxa exhibit a similar level of aggressiveness and have different aggressiveness alleles compared with those originating from domesticated grain legumes? In this review we propose an experimental framework aimed at gaining answers to some of the above questions. The proposed approach includes comparative epidemiology of wild vs. domesticated plant communities, co-evolutionary study of pathogens and their hosts, phenotypic and genetic characterization of fungal isolates from wild and domesticated origins, and genetic analyses of pathogenicity and parasitic fitness among progeny derived from crosses between isolates from wild and domesticated hosts.

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Introduction

Plant pathogens are a major evolutionary force operating in natural ecosystems and in domesticated plant communities (Burdon 1987). In natural ecosystems, both hosts and their pathogens survive but the situation hardly takes the form of an epidemic. Hence, it has long been recognized that the severe epidemics that devastate crop

plants are largely artefacts of cultivation and a result of co-evolution under domestication (Harlan 1976; Burdon 1987). Therefore, gaining better understanding of host–pathogen interaction in its natural state may improve our understanding of the situation in man-made habitats.

The primary and secondary centres of origin of cultivated plants are often also the places of origin of their pathogens (Leppik 1970). Therefore, the Near Eastern cradle of agriculture (Lev-Yadun et al. 2000), where the wild progenitors of the Near Eastern crops and their con-generic taxa co-exist in natural plant formations, may hold some clues on the biology of the pathogens of the respective crops. Near Eastern farming began with the adoption of a small number of crop plants (Zohary and Hopf 2000). This 'Founder Crops' package included einkorn wheat, emmer wheat, barley, lentil, pea, chickpea, bitter vetch, and flax (Zohary and Hopf 2000). At a later stage, additional plants were added to this package including clovers, vetches, medics, fruit trees and vegetables. The farming-based economy spread from the Near East into Europe, Central and East Asia, North and East Africa, and in recent times also into the New World (Diamond 1997). Naturally, wherever the ecological conditions allow, the pathogens of the respective crop plants followed suit. For example, Ascochyta blight pathogens were detected both in the USA and Australia a few years after large scale production of chickpea and lentils was established in these countries and are now considered a major agronomic problem (e.g., Kaiser 1997).

Unlike the situation described above for the USA or Australia, domesticated crop plants grow sympatrically with their wild relatives in the east Mediterranean, (Harlan and Zohary 1966; Zohary 1973). Whenever crop plants grow adjacent to natural ecosystems harbouring stands of wild forms, gene flow between the cultigens and their wild relatives is possible. Indeed, many such examples were described, e.g., for beans in Mexico (Zizumbo-Villarreal et al. 2005), sorghum in Israel and rice in India (Abbo and Rubin 2000). In theory, similar processes can occur between the pathogen populations that exist in such sympatric cropping systems. However, to the best of our knowledge, despite old

reports that Ascochyta pathogens occur on wild legumes in Israel (e.g., Barash 1960), the genetic affinities between the Ascochyta pathogens of the Near Eastern legumes and their relatives infecting the wild forms were hardly studied. In this review we address the Cicer/Ascochyta blight system in wild and in man-made ecosystems (cultivation) as a test case for other Ascochyta pathosystems and flag knowledge gaps relevant for better understanding of the underlying host–pathogen interaction.

Evolutionary, agronomic and ecological considerations

Wheat, barley, pea, lentil and flax spread in pre-historic times around the Mediterranean and into the temperate regions of Europe (Zohary and Hopf 2000). Chickpea, however, took a different pattern compared with the spread of the other Founder Crops and spread across the Mediterranean, but mainly to the south and south-east. Chickpea became a major crop in East Africa and India, mainly as a post-rainy season crop (Ladizinsky 1995) but not in the wheat-based temperate systems of Europe (Ladizinsky 1995; Kumar and Abbo 2001). All Founder Crops species except chickpea have retained their autumnal germination—summer maturation cycle, while across the Near East, traditionally, chickpea is a spring-sown crop (Kumar and Abbo 2001). It was suggested that this crop cycle change from autumn to spring sowing was driven by the extreme vulnerability of chickpea to Ascochyta blight during the rainy season (Abbo et al. 2003).

Ascochyta blight is a fungal disease caused by Didymella rabiei (anamorph: Ascochyta rabiei). It is one of the most important diseases of chickpea worldwide (Nene 1982; Nene and Reddy 1987; Akem 1999) affecting all above ground parts of the plants. Under environmental conditions that favour development of the pathogen, the disease is devastating. Crops are destroyed and yield losses reach 100% (Nene 1984; Akem 1999). Like many other pycnidial fungi, the pathogen spreads during the growing season mainly by rain-splash of pycnidiospores (Nene 1984; Fitt et al. 1989). Although temperature and wind influence disease

development and spread, rainfall is the environmental parameter governing Ascochyta blight epidemics and the disease develops whenever there are rains during the cropping season (Nene and Reddy 1987; Reddy et al. 1990; Diekmann 1992; Akem 1999).

Lentil and pea, two other grain legumes of Near Eastern origin, are also infected by Ascochyta blights. In lentil, the causal agent is *A. lentis* (Kaiser et al. 1997). In pea, the disease is incited by a complex of three pathogens: *A. pisi*, which causes leaf and pod spots; *A. pinodes*, the conidial state of *Mycosphaerella pinodes*, which causes blight; and *Phoma pinodella* (Syn. *Ascochyta pinodella*), which causes foot rot (Ali et al. 1994). Interestingly, Ascochyta blight did not preclude winter sowing of pea or lentil. One possible explanation is the difference in the influence of the disease on these crops. Whereas severe Ascochyta blight epidemics in chickpea are devastating, effects of the disease in lentil and pea are less conspicuous. Although substantial yield losses may occur in pea and lentil, complete destruction of the plants is uncommon even under severe epidemics (Gossen and Morrall 1983; Bayaa et al. 1992; Ali et al. 1994; Tivoli et al. 1996; Morrall 1997).

Is the difference between the aggressiveness/ virulence of the chickpea Ascochyta pathogen and those of lentil and pea related to the different cropping practices? Study of the wild barley/ powdery mildew system in Israel demonstrated that higher aggressiveness and wider virulence range are common in sites where the climatic conditions are unfavourable for the pathogen (Dinoor and Eshed 1987). Likewise, the summer cropping system of chickpea is less favourable to the Ascochyta pathogen than the winter cropping of lentil and pea or the autumn–winter development of wild *Cicer* to their respective Ascochyta pathogens. Could this be the reason for the extreme aggressiveness of Ascochyta in domesticated chickpea fields? Will lower aggressiveness be found in wild populations similar to the above? Contrary to the well-documented situation in the cereals and many of their pathogens (e.g., Dinoor 1974; Dinoor and Eshed 1984; Dinoor et al. 1991), hardly any information is available on the role of fungal pathogens in populations of wild relatives of Near Eastern legumes. Specifically, regarding

chickpea, up to date, the only published report of *D. rabiei* from wild *Cicer* was from the perennial *C. montbretti* in Bulgaria (Kaiser et al. 1998).

Recently however, Frenkel et al. (2007) described the isolation of two Ascochyta pathogens from *C. judaicum*, an annual wild relative of domesticated chickpea native to Israel, Jordan and neighbouring countries. The pathogens, *D. rabiei* and *P. pinodella*, were identified morphologically and the DNA sequences of the rDNA intergenic regions were used to verify the morphological identification according to their similarity with published sequence information (Frenkel et al. 2007). The infectivity of the isolates obtained from the wild was verified by following Koch's postulates. *Didymella rabiei* isolates from wild *C. judaicum* were capable of infecting a number of annual *Cicer* species including domesticated chickpea, its wild progenitor *C. reticulatum*, and *C. bijugum* from Turkey. Disease severity caused by isolates from *C. judaicum* was greater on the wild hosts compared with the domesticated host. Similarly, using isolates originating from domesticated fields resulted in higher disease severity on domesticated cultivars compared with wild *C. judaicum* accessions (Frenkel et al. 2007). Although *P. pinodella* is not the focus of this review, it is interesting to note that this pathogen, which is one of the fungi that compose the Ascochyta complex of pea, also attacks *C. judaicum*. *Phoma pinodella* isolates from *C. judaicum* were able to infect both wild and domesticated peas (*Pisum sativum* and *P. fulvum*, respectively). In the studied ecosystems, wild chickpea grow side by side with wild pea species, and both are within meters from farmland where archaeological remains testify for millennia of cultivation (Frenkel et al. 2007). Such sympatric cropping (and patho-systems) may provide better understanding of the biology of the pathogens and their interaction with wild and domesticated hosts.

Important knowledge gaps

A number of questions emerge from the above description. What is the role of genetic diversity at resistance loci of the wild hosts and is it greater

compared with the cultigen? Do natural and agricultural ecosystems function as independent pathosystems? Specifically, are populations of *D. rabiei* infecting wild *Cicer* genetically distinct from populations infecting domesticated chickpea, and if so, is this differentiation related to differences in host specialization or to adaptation to different ecological conditions? Do isolates sampled from wild *Cicer* exhibit a similar level of aggressiveness and have different aggressiveness alleles compared with those sampled from domesticated chickpea? And last but not least, can we use gene diversity measures of the pathogen to infer about its origin and past and recent migration patterns?

Proposed framework for progress and bearing for resistance breeding

Clarifying the unresolved issues above, and answering the relevant research questions, require extensive multi disciplinary experimental work.

Comparative epidemiology

Modelling approaches are often used to elucidate the influence of environmental parameters on epidemic outbreaks. This was done in domesticated chickpea (e.g., Jhorar et al. 1997), but not in wild *Cicer* populations. Wild *Cicer* populations differ from domesticated plant communities in terms of their physical structure and genetic constitution. Therefore, disease prevalence, spread and development in time and space in the wild are likely to be different from those occurring in farmers' fields. Application of modelling approaches will enable quantification of the association between climatic parameters and disease development characteristics in wild populations. This in turn will point to the differences, if such occur, between the selection pressures operating on the pathogens and their hosts, in natural vs. man-made agro-systems.

Phenotypic and genetic characterization of hosts and pathogens

The different seasonality of wild vs. domesticated chickpea may suggest that pathogen populations parasitizing wild chickpea have different ecological requirements than those infecting domesticated crops. For example, the optimal temperature for spore germination, penetration, establishment and formation of pycnidia and pycnidiospores may differ between pathogen populations from wild and domesticated origins. Comparing the effect of temperature on the components of the disease cycle of isolates originating from wild and domesticated plants will clarify if such differences do exist and what is their magnitude. Similarly, effects of other environmental parameters (such as relative humidity, wetness duration, etc.) can be studied. Analyzing the segregation of the respective phenotype among cross progeny between wild and domesticated isolates will enable the study of the genetic control of the respective traits. Challenging wild *Cicer* accessions and domesticated chickpea with *D. rabiei* isolates from both hosts under controlled conditions may clarify the role of genetic resistance in natural *Cicer* populations. Comparing population structure of the pathogens isolated from wild and domesticated hosts using neutral DNA markers will allow estimation of gene flow among populations on different hosts and between geographic regions. Such analyses will determine if wild *Cicer* populations provide a significant source of inoculum for Ascochyta blight epidemics of domesticated chickpea.

Host–pathogen specialization in the *Cicer*/*D. rabiei* system is another unresolved issue. Some authors refrain from using the term 'race' for *D. rabiei* isolates thereby implying incomplete specialization of the fungus (Lichtenzveig et al. 2005), while others use a race classification of this pathogen (Santra et al. 2000). Several groups have reported significant cultivar-by-isolate interaction (Chen et al. 2003; Chongo et al. 2004; Phan et al. 2003; Cho et al. 2004). Another approach was to define pathotype groups in *D. rabiei* to describe shifts in the pathogen populations that caused breakdown of resistant cultivars (e.g., Reddy and Kebbabeh 1985; Udupa et al. 1998). This yet unresolved debate regarding host–pathogen specialization in the *Cicer*/*D. rabiei* system has important implications for resistance breeding and may benefit from re-evaluation of current breeding strategies as well as disease assessment

methodologies (e.g., use of parametric scales to evaluate disease severity). If host specialization of the pathogen is the rule, it implies that breeders will have to frequently recruit new alleles and maybe even new resistance genes to combat new emerging virulent pathogen genotypes. If, however, host specialization is not a major feature of the pathogen, it may be possible to use existing resistance sources for a longer period like the Israeli cv. Bulgarit (Lichtenzveig et al. 2005). As some collections of Ascochyta isolates are heavily biased towards isolates originating from cultivated fields (e.g., WJ Kaiser collection in Pullman WA, USA), assessment of host specialization among *D. rabiei* requires larger sampling of isolates from the wild and challenging a larger representative collection of wild and cultivated hosts with both domesticated and wild isolates.

Host–pathogen coevolution

Molecular study of Ascochyta pathogens isolated from a number of legume species (wild and domesticated) enabled the assessment of the phylogenetic relationships among the sampled group (Barve et al. 2003; Peever et al. 2006). Like the host plants that undergo speciation processes on an evolutionary time scale, the pathogens are adjusting themselves to the evolutionary trends of their hosts as expected from the intimate interactions that occur through most of the life cycle of the pathogen. The study of such co-evolutionary trajectories taken by different members of the legume family and their Ascochyta pathogens may enable the reconstruction of the evolution of the pathogens. For instance, phylogenetic analyses among Ascochyta taxa from different legume species may help answer the question whether the pathogens parasitizing any given legume taxon are monophyletic or polyphyletic, and if the evolution of the pathogens actually reflects the evolutionary history of their hosts.

Phylogenetic analyses using DNA markers may expose spatial and temporal patterns of population dynamics across large geographical scales (Stukenbrock et al. 2006). Indeed, using DNA markers and hierarchical analyses enabled Zaffarano et al. (2006) to excluded both the Near Eastern cradle of agriculture and the Ethiopian

barley diversity centre as the origin of the barley scald pathogen *Rhynchosporium secalis*, due to higher gene diversity found in central Europe. Similarly, study of a world collection of *D. rabiei* enabled Peever et al. (2004) to draw both a recent and a historical picture of population structure of this pathogen in the USA Pacific Northwest. However, due to the extremely small number of isolates from wild chickpea it is impossible at the present time, to hypothesize on the geographical origin of *D. rabiei* infecting domesticated chickpea. Study of Ascochyta isolates with special emphasis on isolates from wild *Cicer* both within the Near Eastern cradle of agriculture as well as in areas outside the natural distribution of the hosts, may expose the historical trends of the spread of the pathogen, and may enable the detection of the centre of origin of current pathogens or sources of recent epidemic episodes (e.g., epidemic outbreak in Australia in the late 1990s). Such analysis will also enable the refutation or corroboration of the hypothesis of Abbo et al. (2003) regarding Bulgaria and perennial *C. montbretii* as a possible origin of *D. rabiei* infecting domesticated chickpea. Such information is important since areas of maximum gene diversity of the pathogen are also likely to be important sources of host resistance genes, both wild and domesticated. In addition, identification of migration patterns and direction of gene flow in the pathogen may help in devising better quarantine measures within as well as between continents for the benefit of farmers worldwide.

Genetic analysis of pathogenicity

The quantitative nature of the *Cicer/D. rabiei* interaction suggests polygenic control of resistance/aggressiveness. Evidence for quantitative resistance in the host was published (e.g., Santra et al. 2000; Tekeoglu et al. 2000; Lichtenzveig et al. 2002; Flandez-Galvez et al. 2003; Lichtenzveig et al. 2006) but we currently lack data concerning the genetic control of aggressiveness in the pathogen. Such information could be obtained from the genetic analysis of progeny derived from crosses between isolates with different aggressiveness phenotypes on wild and domesticated hosts. Application of quantitative

genetic tools, in conjunction with DNA markers (e.g., Lichtenzveig et al. 2002, 2006) and phenotypic assessment (e.g., above) will enable the determination of whether aggressiveness genes are genetically linked to loci governing ecological adaptation (e.g., temperature- or wetness-response loci). This may help in answering the question: what is the role of environmental determinants in the co-evolution of resistance/aggressiveness in the *Cicer/D. rabiei* pathosystem.

Concluding remarks

A combination of factors determines that host/pathogen co-evolution under domestication is likely to follow a different trajectory compared with the situation in natural ecosystems. Among the factors relevant to the *Cicer/D. rabiei* pathosystem are: plant density (dense vs. thin), the genetic structure of host populations (uniform vs. variable), seasonal profile (warmer and drier vs. colder and wetter) under cultivation and in natural ecosystems, respectively. Therefore, study of (domesticated) biased collections of fungal isolates and their interaction with domesticated cultivars is unlikely to expose the full spectrum of the host–pathogen interaction in the respective pathosystem (Harlan 1976). This, in turn, might limit our ability to develop effective management strategies or efficient breeding methodology (see above). To complement the partial picture obtained from the study of domesticated host–pathogen interactions, the above experimental approach is proposed. It is anticipated that recent initiatives taken by the present authors and other groups to study the ecology and the genetics of the respective legume sympatric pathosystems will provide plant breeders, agronomists and pathologists with better tools for more effective disease management.

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REVIEW PAPER

Role of host specificity in the speciation of *Ascochyta* pathogens of cool season food legumes

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Abstract *Ascochyta*/legume interactions are attractive systems for addressing evolutionary questions about the role of host specificity in fungal speciation because many wild and cultivated cool season food legumes are infected by *Ascochyta* spp. and most of these fungi have described teleomorphs (*Didymella* spp.) that can be induced in the laboratory. Recent multilocus phylogenetic analyses of a worldwide sample of *Ascochyta* fungi causing ascochyta blights of chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), and pea (*Pisum sativum*) have revealed that fungi causing disease on each host formed a monophyletic group. Host inoculations of these fungi demonstrated that they were host-specific, causing disease only on the host species from which they were isolated. In contrast to the strict association between monophyletic group and host observed for pathogens of cultivated legumes, *Ascochyta* fungi causing disease on wild bigflower vetch (*Vicia grandiflora*) were polyphyletic. Genetic crosses between several pairs of closely related, host-specific, and phylogenetically distinct *Ascochyta* fungi were fully sexually compatible. Progeny from these crosses had normal cultural morphology and segregation of molecular markers indicating a lack of intrinsic, post-zygotic mating barriers between the

parental taxa. However, when progeny from a cross between a faba bean-adapted isolate (*A. fabae*) and a pea-adapted isolate (*A. pisi*) were assessed for their pathogenicity to the parental hosts, almost all progeny were non-pathogenic to either faba bean or pea. These results suggest that although these fungi have retained the ability to mate and produce progeny with normal saprophytic fitness, progeny are severely compromised in parasitic fitness. The host specificity of these fungi, coupled with the inability of hybrid progeny to colonize and reproduce on a host, may constitute strong extrinsic, pre-zygotic and post-zygotic mating barriers in these fungi and promote the genetic isolation and speciation of host-specific taxa. A phylogeny of the host plants is also being developed, and with more extensive sampling of pathogens and hosts from sympatric populations in the centre of origin, the hypothesis of cospeciation of pathogens and hosts will be tested. The objectives of this review are: (1) to summarize recent phylogenetic, host specificity and speciation studies of *Ascochyta* fungi, and (2) to suggest how current and future research using these pathosystems may lead to a better understanding of the role of host specificity in the speciation of plant-pathogenic fungi and the cospeciation of pathogens and their hosts.

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Biology of *Ascochyta* spp.

Species of the coelomycete genus *Ascochyta* infect a number of economically important cool season food legumes and the diseases they cause represent serious limitations to legume production worldwide. Well-known hosts include chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), pea (*Pisum sativum*), vetches (*Vicia* spp.) and their wild relatives. These diseases are known as ascochyta blights and are characterized by tan-coloured lesions on all above-ground parts of the plant which contain concentric rings of black pycnidia exuding cirrhi of one or two-celled hyaline conidia (Nene and Reddy 1987). Conidia are dispersed short distances via rain-splash and are responsible for secondary disease cycles during the growing season of the crop (Nene and Reddy 1987; Kaiser 1992). Some species of *Ascochyta* also reproduce sexually and ascospores can be windborne and dispersed long distances by air (Trapero-Casas et al. 1996). Ascospores are typically unequally two-celled with a prominently constricted septum (Wilson and Kaiser 1995; Kaiser et al. 1997) and are considered important sources of primary inoculum in areas where both mating types occur (Trapero-Casas et al. 1996; Kaiser 1997a, b; Peever et al. 2004). Most *Ascochyta* spp. have a bipolar, heterothallic mating system (Barve et al. 2003; Cherif et al. 2006). *Ascochyta* spp. have been moved by human activity into most areas of the world where cool season food legumes are currently produced (Morrall and McKenzie 1974; Kaiser 1997a, b; Peever et al. 2004). Most of this movement has been due to the introduction of infected and/or infested seed imported for agronomic evaluation (Kaiser 1992; Peever et al. 2004). *Ascochyta* fungi have been demonstrated to be seedborne (Kaiser 1972; Morrall and McKenzie 1974; Maden et al. 1975) and have the potential to be transmitted from seed to seedling (Maden 1983; Dey and Singh 1994; Kimber et al. 2006). Cool season food legumes are native to south-eastern Turkey, Iran, Iraq, and Syria (Van Der Maesen 1987; Smartt 1990) and we hypothesize that *Ascochyta* spp. coevolved with their hosts in these areas.

Species of *Ascochyta*

Ascochyta fabae, A. pisi, A. lentis, A. rabiei, and *A. viciae-villosae* are pathogens of faba bean (*V. faba*), pea (*P. sativum*), lentil (*L. culinaris*), chickpea (*C. arietinum*) and hairy vetch (*Vicia villosa*), respectively (Nene and Reddy 1987; Nene et al. 1988; Kaiser 1997a, b; Mel'nik et al. 2000). Several *Ascochyta* anamorphs have been connected to *Didymella* teleomorphs (Jellis and Punithalingam 1991; Kaiser et al. 1997). The taxonomy of *Ascochyta* spp. is based first on morphological characters such as the shape and size of conidia, conidial septation, and second on host of isolation and molecular markers (Gossen et al. 1986; Kaiser et al. 1997; Fatehi and Bridge 1998). *Ascochyta rabiei*, the chickpea pathogen, grows approximately five times more slowly in culture and has darker colony morphology compared to *A. lentis, A. fabae,* and *A. pisi*. Genetic crosses made between *A. rabiei* and A. *fabae* and between *A. rabiei* and *A. lentis* failed to produce any pseudothecia while crosses between *A. fabae* and *A. lentis* produced pseudothecia (Kaiser et al. 1997). Results of these crosses predict that *A. fabae* and *A. lentis* are more closely related to each other than either is to *A. rabiei*. *Ascochyta fabae, A. lentis, A. viciae-villosae* and *A. pisi* are morphologically similar and have been historically difficult to separate using morphological criteria alone. Efforts to differentiate *A. fabae* and *A. lentis* provide an interesting case study in fungal species concepts. Gossen et al. (1986) demonstrated that *Ascochyta* spp. isolates from lentil and faba bean only caused disease on lentil and faba bean, respectively. However, these host-specific taxa could not be differentiated by statistical analyses of conidium length, proportion of septate conidia and cultural morphology (Gossen et al. 1986). These authors proposed that these two fungi be synonymized under *A. fabae* using the *formae speciales* designations *A. fabae* f.sp. *fabae* and *A. fabae* f.sp. *lentis* to denote their morphological similarity and host specificity. Crosses of these taxa were fertile and produced pseudothecia with viable ascospore progeny (Kaiser et al. 1997) but strong post-zygotic mating effects were observed which included abnormal numbers of ascospores in each ascus, variable ascospore size, and progeny isolates that grew abnormally in culture. In addition, all progeny isolates failed to infect either of their parental hosts. Kaiser et al. (1997) also scored these isolates for RAPD-PCR markers and showed that the fungi from each host each had distinct RAPD-PCR banding profiles and clustered separately in a UPGMA phenogram. The combination of host

specificity, strong genetic differentiation in molecular markers (i.e., lack of gene flow) and post-zygotic mating effects observed in this study were used to justify the elevation of *A. fabae* f.sp. *lentis* to *A. lentis* Vassilevsky (Kaiser et al. 1997), and represents a rare example of application of the biological species concept to plant-pathogenic fungi. Currently, we consider *A. rabiei*, *A. fabae* and *A. lentis* to be well-supported biological and/or morphological species.

Evolutionary relationships among *Ascochyta* spp.

Despite the economic importance of the cool food season legumes and the *Ascochyta* spp. that cause devastating losses of these plants, little is known about the evolutionary history of either the hosts or the pathogens. Peever et al. (2007) recently estimated phylogenies among the *Ascochyta* spp. pathogens of the cool season legumes using DNA sequence data from several regions of the genome including a glyceraldehyde-3-phosphate dehydrogenase gene (*G3PD*), a chitin synthase 1 gene (*CHS*) and translation elongation factor 1 alpha gene (*EF*). The analysis employed an extensive collection of *Ascochyta* spp. from cool season food legumes established by W. J. Kaiser, USDA-ARS and maintained at Washington State University. This collection contains isolates from chickpea, pea, lentil, hairy vetch and faba bean sampled on a worldwide scale. Currently, the collection is biased heavily towards fungi from cultivated legumes, but has been augmented in recent years with isolates sampled from wild legume species including some of the closest known relatives of cultivated crops. Isolates from wild legumes have been obtained during collecting trips to Armenia, the Republic of Georgia and Spain. Phylogenetic analyses of the combined *G3PD*, *CHS* and *EF* datasets using maximum likelihood methods revealed that *A. rabiei*, the pathogen of chickpea (*C. arietinum*), was distinct from the *Ascochyta* pathogens of pea, faba bean, wild vetches and lentil which were found in two differentiable but closely related clades (Fig. 1, Peever et al. 2007). The differentiation observed between *A. rabiei* and *A. lentis*/*A. fabae* in the combined phylogeny correlates well with the results of genetic crosses among these same taxa made previously (Kaiser et al. 1997). The combined phylogeny also revealed that isolates

sampled from wild *Cicer* spp. (*C. montbretii* and *C. ervoides*) had sequences that were identical or nearly identical to isolates from cultivated chickpea (*C. arietinum*). *Cicer arietinum* is an annual species which is genetically distinct from the perennial species, *C. montbretii* and *C. ervoides* (Javadi and Yamaguchi 2004; Sudupak et al. 2004). The genetic similarity of fungi colonizing distantly related annual and perennial *Cicer* hosts suggests that the source of the ascochyta blight fungus for epidemics on cultivated chickpea may be wild, perennial chickpea relatives.

Two major clades were apparent in the combined phylogeny, one corresponding to isolates from cultivated lentil (*L. culinaris*), hairy vetch (*V. villosa*) and wild *Vicia* spp. (the *A. lentis*/*A. viciae-villosae* clade) and one corresponding to isolates from cultivated pea (*P. sativum*) and faba bean (*V. fabae*), wild pea (*P. elatius*) and wild *Vicia* spp. (the *A. fabae*/*A. pisi* clade) (Fig. 1). Isolates sampled from wild legume hosts displayed more sequence variation for all genomic regions compared to isolates from cultivated hosts (Fig. 1) consistent with the hypothesis that *Ascochyta* spp. pathogens of cultivated legumes represent a subset of the variation present in pathogen populations on wild hosts. More intensive sampling of *Ascochyta* spp. from sympatric legume hosts in the centre of origin will be required to definitively test this hypothesis. Isolates sampled from the cultivated hosts lentil, pea, faba bean, and chickpea were each monophyletic with strict correlation between phylogenetic clade and host of origin. In contrast, isolates from the wild hosts *V. villosa* and *V. lathyroides* formed a well-supported sub-clade within the *A. lentis* clade and isolates from *V. grandiflora* and *V. cordata* and from *V. grandiflora* and *V. sepium* also formed well-supported sub-clades within the *A. fabae*/*A. pisi* subclade (Fig. 1). Perhaps the most interesting result of the phylogenetic analysis was that isolates sampled from wild *V. grandiflora* were polyphyletic, distributed in three clades (Fig. 1). There are at least two hypotheses that may explain the polyphyly of the fungi sampled from this host. The first is that *V. grandiflora* may be colonized by different evolutionary lineages of *Ascochyta* pathogens. This would imply that the apparent tight correlation between pathogen clade and host of origin seen with isolates from cultivated hosts is the result of a founder event or strong selection by each
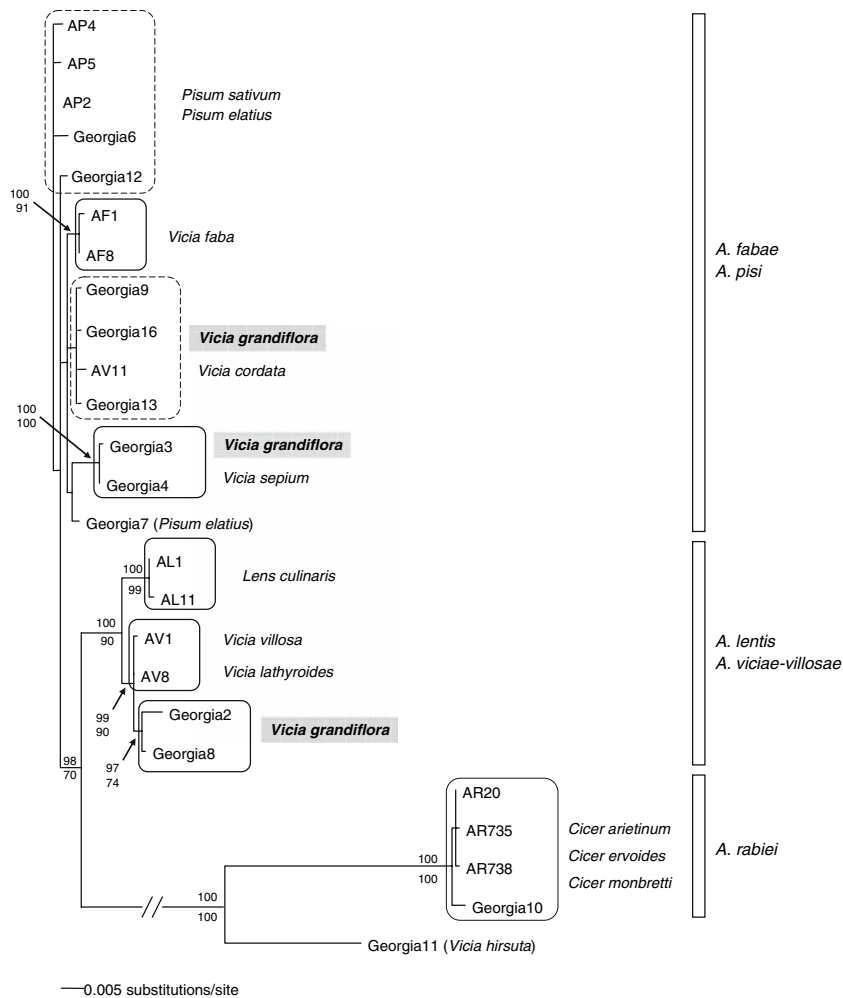
Fig. 1 Maximum likelihood phylogeny estimated from the combined chitin synthase (*CHS*), translation elongation factor alpha (*EF*) and glyceraldehyde-3-phosphate-dehydrogense (*G3PD*) datasets for *Ascochyta* and *Didymella* spp. sampled from various legume hosts. Upper numbers at major nodes indicate Bayesian posterior probabilities of sampling the node among 6,000 trees (600,000 generations of the MCMC chain) and lower numbers indicate percent ML bootstrap values from 1,000 bootstrapped datasets. Clades were inferred based on ML bootstrap values greater than or equal to 70% and posterior probabilities greater than or equal to 95%. Major clades are identified by open vertical bars and well-supported sub-clades by solid-line boxes. Clades with bootstrap values and posterior probabilities below the significance criteria are indicated by dashed-line boxes. Branch lengths are proportional to the inferred amount of evolutionary change and the scale represents .01 nucleotide substitutions per site. Host of isolation is indicated to the right of the taxon labels and isolates sampled from *Vicia grandiflora* are indicated in grey

cultivated host for a single monophyletic lineage of fungus and possibly coevolution of host and pathogen. The alternative hypothesis is that *Ascochyta* fungi causing disease on both wild and cultivated hosts have tight coevolutionary relationships with their hosts but the host taxa identified in this study are polyphyletic. The host plants sampled in our study were all identified morphologically in the field and it is possible that several distinct evolutionary lineages

were classified as *V. grandiflora*. In order to distinguish between these two hypotheses, more detailed sampling in the centre of origin and more careful morphological analysis of the hosts needs to be performed as well as controlled inoculations of hosts and phylogenetic analyses of the hosts based on DNA sequence data.

Isolates from wild pea (*P. elatius*), the presumed ancestor of cultivated pea (Smartt 1990), clustered

with isolates from cultivated pea (*A. pisi*) in the *A. fabae/A. pisi* clade (Fig. 1). This is consistent with the hypothesis that *A. pisi* on cultivated pea evolved on *P. elatius* or other wild relatives, becoming a pathogen of pea during its domestication. Preliminary host inoculations have demonstrated that isolates of *A. pisi* sampled from cultivated pea are able to cause disease on both cultivated and wild pea but that isolates from wild pea are only able to cause disease on wild pea (T. Horton, M.I. Chilvers and T.L. Peever, unpublished). These data, although preliminary, may indicate that certain genotypes of the pathogen have a wider host range that allowed an expansion of host range during the domestication of pea. Crosses between isolates that are exclusively pathogenic on wild pea and isolates capable of inducing disease on both wild and cultivated pea may provide insight into the genetic control of host range and the mechanism responsible for this difference in host range.

Host specificity and speciation of *Ascochyta* spp.

Artificial inoculations in the greenhouse and in growth chambers have demonstrated that legume-associated *Ascochyta* fungi are host-specific (Kaiser 1973; Tripathi et al. 1987; Kaiser 1991; Kaiser et al. 1997; Khan et al. 1999; Hernandez-Bello et al. 2006). *Ascochyta fabae*, *A. pisi*, *A. rabiei*, *A. lentis* and *A. viciae-villosae* caused disease when inoculated onto faba bean, pea, chickpea, lentil and hairy vetch, respectively (Hernandez-Bello et al. 2006). The results of Hernandez-Bello et al. (2006) agree with previous inoculation studies where *A. rabiei* failed to cause disease on lentil, pea and vetch (Kaiser 1973; Tripathi et al. 1987; Kaiser 1991; Khan et al. 1999) and *A. fabae* and *A. lentis* could only cause disease on their respective hosts (Kaiser et al. 1997). The phylogenetic analyses demonstrated that *A. rabiei*, *A. pisi*, *A. lentis*, *A. fabae*, and *A. viciae-villosae* are each monophyletic (Fig. 1). These taxa are also host-specific (Hernandez-Bello et al. 2006). *Ascochyta pisi* is most closely related to *A. fabae* and *A. lentis* is most closely related to *A. viciae-villosae* (Fig. 1). Crosses made between these pairs of host-specific taxa were fertile and did not appear to have any of the genetic abnormalities observed in the crosses between *A. fabae* and *A. lentis* made previously by Kaiser et al. (1997). Interspecific hybridization of *A.*

pisi × *A. fabae* and *A. viciae-villosae* × *A. lentis* was confirmed by the segregation of mating type and molecular markers. Segregation ratios of amplified fragment length polymorphism (AFLP) markers in these interspecific crosses were not significantly more distorted when compared to intraspecific crosses (Hernandez-Bello et al. 2006) demonstrating a lack of obvious intrinsic postzygotic mating defects. Both crosses produced viable ascospore progeny with normal cultural morphology and growth rates. However, artificial inoculations of progeny isolates from the *A. pisi* × *A. fabae* cross in the greenhouse and growth chamber resulted in very few progeny that were able to induce disease on either parental host. These data indicate that *A. fabae* and *A. pisi* are closely related phylogenetic species, can be experimentally crossed and that host specificity is likely to be polygenic. These data also suggest that fitness deficits suffered by the progeny of such a cross (i.e. the inability to cause disease and reproduce on a host plant) may be high and contribute a strong mating barrier. The results of the inoculation study with progeny from the *A. fabae* × *A. pisi* cross (Hernandez-Bello et al. 2006) were similar to those reported by Kaiser et al. (1997) for the much wider *A. fabae* × *A. lentis* cross. Mechanisms of speciation of fungi are poorly understood but host specificity may play an important role in facilitating the speciation of *Ascochyta* spp. and other host-specific, plant pathogenic fungi (Kohn 2005; Giraud et al. 2006). It is possible that host specialization of *Ascochyta* spp. acts as a prezygotic isolating mechanism as in other plant pathogens, including *Phytophthora* spp. (Goodwin and Fry 1994; Goodwin et al. 1999), formae speciales of *Blumeria graminis* (Hiura 1962; Hiura 1978) and *Puccinia graminis* (Johnson 1949). All of our observations, taken together, suggest that host specificity in *Ascochyta* may represent both a prezygotic and a postzygotic mating barrier and that these barriers have played important roles in the speciation of *Ascochyta* fungi. The evolution of host specificity may represent the initial step in the speciation of these fungi. In addition to uncovering the role of host specificity in fungal speciation, crosses between closely related pairs of *Ascochyta* taxa and inoculation of the progeny may allow determination of the genetics of species-level host specificity which is a largely unexplored area in plant pathology (Heath 1991).

Cospeciation of *Ascochyta* spp. and their legume hosts?

The host specificity of closely related *Ascochyta* fungi indicates that this character has likely played an important role in the speciation of these fungi and it is possible that coevolutionary interactions between pathogen and host may have resulted in cospeciation of pathogen and host (Thompson and Burdon 1992). In support of this hypothesis, the combined glyceraldehyde-3-phosphate dehydrogenase (*G3PD*), translation elongation factor 1 alpha (*EF*), and chitin synthase (*CHS*) phylogeny estimated among the *Ascochyta* spp. correlates well with a plastid *matK*

phylogeny of the hosts (Fig. 2) (Steele and Wojciechowski 2003; Steele and Wojciechowski, unpublished). The differentiation seen between *Cicer* spp. (tribe Cicereae) and *Pisum*, *Vicia* and *Lens* spp. (tribe Vicieae) in the host *matK* phylogeny is mirrored by the pathogen phylogeny. Steele and Wojciechowski (2003) identified two subclades within the Vicieae including Clade 1 which contained *P. sativum* and Clade 2 which contained *L. culinaris* and *V. grandiflora*. Although there was not complete overlap in the hosts sampled for the Steele and Wojciechowski (2003) study and our study (Peever et al. 2007), there appears to be broad congruence between pathogen and host phylogenies with the Steele and Wojciechowski

Fig. 2 Phylogeny of Vicioid clade (left) based on parsimony analysis of complete *mat*K gene aligned with combined *CHS*, *EF*, and *G3PD* phylogeny of *Ascochyta* fungi (right-simplified and inverted representation of Fig. 2). Plant tree shown is one representative of 1,000 equally parsimonious trees (1,865 steps), 1,524 characters included (168 excluded of 1,692 total), 485 of which are parsimony informative; CI = .5727, RI = .8444; tree rooted using *Glycyrrhiza* and *Callerya* (not shown). Bootstrap proportions shown near nodes for all nodes resolved in strict consensus tree; support for larger clade, the IRLC = 100%. Each host species is colour-coded and black lines connect legume hosts and fungi isolated from those same hosts. Orange lines illustrate polyphyly of *Ascochyta* fungi isolated from *Vicia grandiflora*
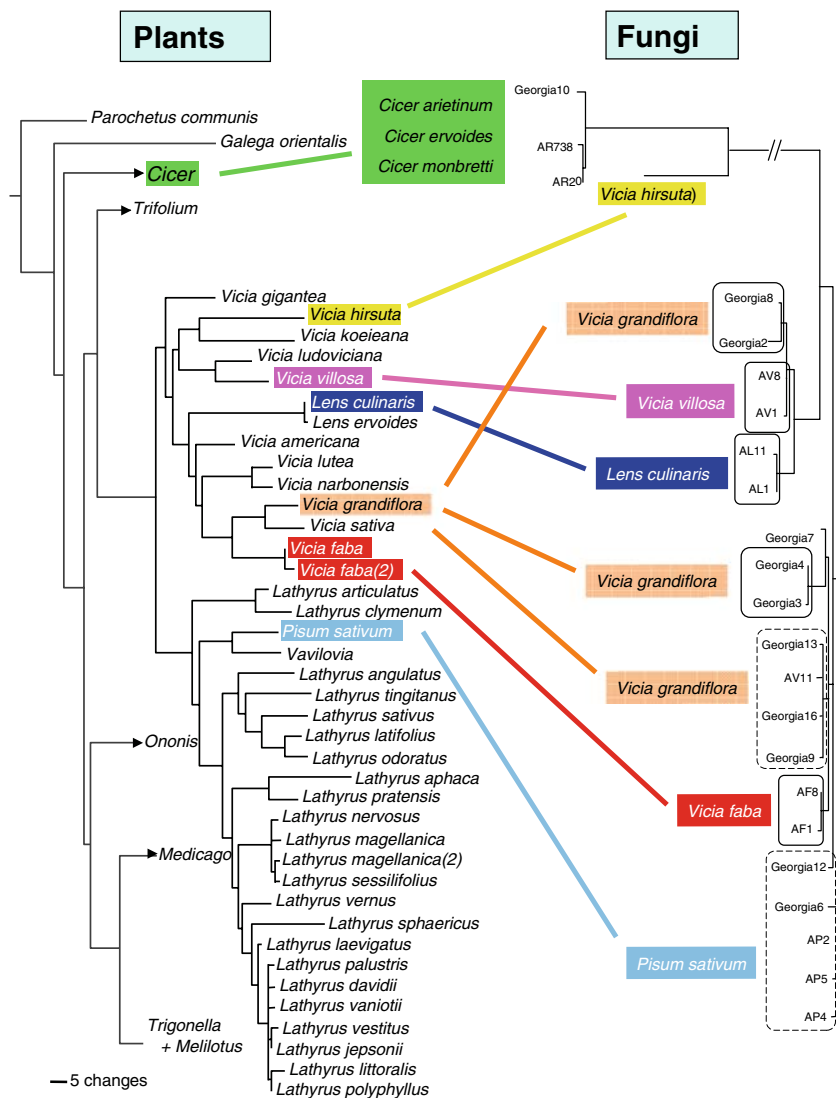
(2003) Clade 1 corresponding to the *A. pisi/A. fabae* clade in the combined analysis reported here and the Steele and Wojciechowski (2003) Clade 2 corresponding to the *A. lentis* clade reported here (Fig. 2). In order to rigorously test the cospeciation hypothesis, more extensive sampling of pathogen and host from sympatric host populations in the centre of origin are required. Cospeciation analyses will also require lower-level phylogenetic and phylogeographic analyses of the host using faster-evolving regions of the genome. Statistical tests of congruence between robust pathogen and host phylogenies will allow critical tests of cospeciation (Paterson and Banks 2001). Additional fast-evolving regions of the legume genome have been identified and are currently being used to resolve the evolutionary relationships within the Vicieae and Cicereae tribes (Steele and Wojciechowski 2003) and these regions will be useful for resolving phylogenetic relationships among closely related hosts. Sampling of *Ascochyta* fungi from sympatric hosts in these tribes in their centre of origin coupled with estimation of robust lower-level phylogenies for both hosts and pathogens will provide interesting insights into the coevolution of these pathosystems.

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REVIEW PAPER

Diagnostics, genetic diversity and pathogenic variation of ascochyta blight of cool season food and feed legumes

Paul W. J. Taylor · Rebecca Ford

Abstract Molecular diagnostic techniques have been developed to differentiate the *Ascochyta* pathogens that infect cool season food and feed legumes, as well as to improve the sensitivity of detecting latent infection in plant tissues. A seed sampling technique was developed to detect a 1% level of infection by *Ascochyta rabiei* in commercial chickpea seed. The *Ascochyta* pathogens were shown to be genetically diverse in countries where the pathogen and host have coexisted for a long time. However, where the pathogen was recently introduced, such as *A. rabiei* to Australia, the level of diversity remained relatively low, even as the pathogen spread to all chickpea-growing areas. Pathogenic variability of *A. rabiei* and *Ascochyta pinodes* pathogens in chickpea and field pea respectively, appears to be quantitative, where measures of disease severity were based on aggressiveness (quantitative level of infection) rather than on true qualitative virulence. In contrast, qualitative differences in pathogenicity in lentil and faba bean genotypes indicated the existence of pathotypes of *Ascochyta lentis* and *Ascochyta fabae*. Therefore, reports of pathotype discrimination based on quantitative differences in pathogenicity in a set of specific genotypes is questionable for several of the ascochyta-legume pathosystems such as *A. rabiei* and *A. pinodes*. This is not surprising since host resistance to these pathogens has been reported to be mainly quantitative, making it difficult for the pathogen to overcome specific resistance genes and form pathotypes. For robust pathogenicity assessment, there needs to be consistency in selection of differential host genotypes, screening conditions and disease evaluation techniques for each of the *Ascochyta* sp. in legume-growing countries throughout the world. Nevertheless, knowledge of pathotype diversity and aggressiveness within populations is important in the selection of resistant genotypes.

Keywords Diagnostics · Genetic diversity · Pathotypes · *Ascochyta* · *Cicer* · *Lens* · *Pisum* · *Vicia*

Introduction

Ascochyta blight is considered to be one of the most damaging necrotrophic diseases of cool season food and feed legumes worldwide. The disease in chickpea is caused by *Ascochyta rabiei* (teleomorph: *Didymella rabiei*); in lentil by *Ascochyta lentis* (*Didymella lentis*); in faba beans by *Ascochyta fabae* (*Didymella fabae*); and in field pea by *Ascochyta pinodes* (teleomorph: *Mycosphaerella pinodes*), *Ascochyta pisi* and *Phoma medicaginis* var *pinodella*, formerly known as *Ascochyta pinodella*. The pathogens attack

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above ground parts of plants and may produce phytotoxins that lead to necrosis (Tivoli et al. 2006). Knowledge of the biology of ascochyta blight of cool season food and feed legumes will lead to the development and implementation of better control methods for these pathogens. This review will focus on progress that has been made on developing diagnostic techniques to identify the *Ascochyta* species, studying the genetic diversity of the pathogens, and identifying pathogenic variation.

Diagnostics for detection and identification

Spread and development of ascochyta blight diseases can occur through splash and airborne conidia and/or ascospores as well as by commercial distribution of plant material or seeds (Tivoli et al. 2006). Therefore, development of effective disease management depends among others on the rapid detection and precise identification of the pathogen. Traditionally, identification and characterization of fungal species has been based on morphological characters such as size and shape of conidia and appressoria, teleomorph state and cultural characters such as colony colour, growth rate and texture. These criteria alone have not always been adequate due to overlap in morphological characters; and phenotypic variation among related species and under different environmental conditions. To overcome the inadequacies of these traditional techniques, PCR amplification of specific gene sequences has been employed to characterise and analyse the taxonomic complexity of various genera (Faris-Mokaiesh et al. 1996; Phan et al. 2002; Ford et al. 2004). As well, PCR amplification of diagnostic sequences was shown to be highly sensitive to detect small amounts of the organism within the plant tissue, and specific enough to detect only the targeted species (Phan et al. 2002). PCR-based techniques efficiently detect pathogens, especially those that remain latent in the plant tissue and seeds.

PCR techniques based on amplification of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region have been used widely for the differentiation and detection of closely related fungal species (Nazar et al. 1991; Lévesque et al. 1994; Tisserat et al. 1994; Faris-Mokaiesh et al. 1996). Ribosomal genes are suitable for use as molecular probes because of their high copy number. Despite the high conservation between functional rDNA coding sequences, the nontranscribed and ITS regions are usually less conserved and are thus suitable as target sequences for the detection of recent evolutionary divergence.

Using restriction enzyme digestion of PCR-amplified ITS regions of the 18–25S ribosomal genes, Phan et al. (2002) differentiated *A. rabiei*, *A. pinodes*, *A. lentis* and *A. fabae.* However, *A. lentis* and *A. fabae* were identical in this genomic region indicating that they may have diverged more recently than other *Ascochyta* species. Using a similar PCR-RFLP technique, Faris-Mokaiesh et al. (1996) distinguished *A. pisi* from *A. pinodes* and *P. medicaginis* var. *pinodella* but could not differentiate *A. pinodes* from *P. medicaginis* var. *pinodella*. However, Bouznad et al. (1995) was able to separate *A. pisi* from the other two fungi using RAPD analysis. To elucidate the taxonomy further and for a more specific diagnostic tool, less conserved genes such as β-tubulin and the translation elongation factor (TEF) (O'Donnell et al. 1998) should be sequenced for each Ascochyta species.

The PCR test that Phan et al. (2002) developed was able to detect ascochyta blight of chickpea with sensitivity to 0.1 pg of *A. rabiei* genomic DNA. Nevertheless, a diagnostic technique is only as good as the procedure used to sample the population. Strategies need to be developed to increase the likelihood of detecting a low level of infection especially in seed that will be distributed for planting. Phan et al. (2002) developed an efficient method for detecting *A. rabiei* infection in chickpea seed that could be used to assess samples of seed prior to distribution and planting. Samples of chickpea seed (100 per batch) were incubated in a liquid fungal growth medium (Czapek-Dox) for 12–18 h prior to analysis with PCR. The test was successful in detecting a 1% level of infection in commercial chickpea seed samples (Phan et al. 2002). Development of efficient diagnostic techniques to detect latent infection of ascochyta blight pathogens in plant tissue, such as seed, will restrict the importation of more aggressive isolates into countries where cool season food legumes are grown. The development of PCR-based diagnostic tests for the other legume *Ascochyta* species would also be useful for detecting latent infection in seed and help minimize the spread and outbreak of diseases.

Genetic diversity

Knowledge of the genetic diversity of a pathogen population will lead to an understanding of how the pathogen is likely to adapt or evolve to changes in the environment, such as exposure to fungicides and resistant plant genotypes (McDonald and Linde 2002). Genetic diversity can be measured using dominant molecular markers that randomly amplify genomic sequences but provide limited information on diversity between and within populations, or with co-dominant molecular markers that can measure gene flow through allelic variation between populations. Measuring allelic variation can provide an indication of the level of genetic diversity and genetic differentiation that has resulted from evolutionary forces acting on the genes (McDonald and Linde 2002). Breeding programmes can therefore be optimized to screen germplasm for resistance with pathogen isolates that are both representative of the overall genomic variation and the pathogenic variation of the pathogen population.

Ascochyta pathogens are heterothallic since they possess a single mating type locus (*MAT*) with two alternate forms (*MAT1-1* and *MAT1-2*) that must be different for two isolates to mate (Trapero-Casas and Kaiser 1992; Wilson and Kaiser 1995). Sexual recombination within these species may be a potentially significant major factor in determining population structure, as it results in the generation of new and potentially stable genotypes and thus contributes to genetic diversity and adaptive potential (Milgroom 1996; McDonald and Linde 2002).

The *Ascochyta* pathogens have been shown to be genetically quite diverse in many countries where the host and pathogen have co-existed for a long time (Wilson and Kaiser 1995). The level of genetic diversity was found to be quite high in populations of *A. rabiei* isolated from chickpea plants from a broad range of countries (Syria and Lebanon–Udupa et al. 1998; Spain–Navas-Cortes et al. 1998; Pakistan–Jamil et al. 2000; Canada–Chongo et al. 2004). However, the use of dominant molecular markers used in these analyses and in most cases the small sample sizes resulted in a lack of knowledge on the population structures.

Barve et al. (2004) used a specific microsatellite locus to identify a high level of genetic diversity in *A. rabiei* isolates from 16 countries. Analysis of isolates of *A. rabiei* from the USA using AFLP, SSR markers and the mating type locus (*MAT1-1*) indicated that most of the diversity of *A. rabiei* originated from the introduction of a large number of isolates into the USA between 1983 and 1984 (Peever et al. 2004). In a recent study, Rhaiem et al. (2006) found a high level of allelic diversity of SSR loci in *A. rabiei* isolates obtained from five chickpea-growing regions in Tunisia that formed two main sub-populations. Analysis of the *MAT* loci in these populations indicated that *MAT1-2* may have been most recently introduced through two independent introductions.

In contrast, Phan et al. (2003a) and Pradhan (2006) found that in Australia, the genetic diversity among *A. rabiei* isolates collected between 1995 and 2003 was very low when measured using SSR markers compared to the diversity detected among isolates from Tunisia, Syria, Canada and USA. Also, only one mating type (*MAT1-2*) has been detected in Australia despite the discovery of the teleomorph in the field (Galloway and Macleod 2003). Mating type was studied using PCR-based primers specific to the MAT genes (Phan et al. 2003b, Barve et al. 2003). The lack of diversity detected in Australia may reflect a founder effect whereby the pathogen was recently introduced into Australia and then subsequently quickly spread to all chickpea-growing areas, most likely by infected seed. Given that most Australian-grown chickpea genotypes are moderately to highly susceptible to the pathogen, it is reasonable to expect that the pathogen would not have been subjected to selection pressure caused by host resistance. However, the recent release of moderately resistant genotypes may cause greater selection pressure on the pathogen and potentially lead to an increase in variation. Accordingly, the Australian *A. rabiei* population will be monitored closely over the coming seasons for potential changes in genetic diversity, particularly in areas where new resistance sources are sown.

A high amount of genetic diversity was detected with RAPD analysis among Australian *A. lentis* isolates of both mating types (Ford et al. 2000). The isolates most geographically close were most genetically related and a similar level in diversity was detected within Australia as in other lentil-growing regions of the world. In contrast, Onfroy et al. (1999) found very little intraspecific diversity using RAPD analysis among 50 isolates of *A. pinodes* collected from infected field peas grown in France.

Pathogenic variation

Knowledge of pathogenic diversity is important when choosing appropriate isolates to screen for resistance in plant breeding programmes. Many studies have shown pathogenic diversity among isolates within a particular *Ascochyta* species via screening on a set of differential genotypes or cultivars. However, there is concern as to whether true pathotype differences exist or if the differences observed in disease severity are a measure of the natural distribution of aggressiveness within a population, ranging from low to high. A pathotype can be defined as a subclass or group of isolates distinguished from others of the same species by its virulence on a specific host (genotype) i.e., a qualitative difference in disease severity. In contrast, aggressiveness reflects the natural variation in virulence or level of disease (measured quantitatively) within the pathogen population. Often the terminology for pathotypes is interchanged with races, however, an isolate of a pathogen can only be defined as a race when a qualitative difference in virulence occurs where host resistance genes are defined in a set of differential genotypes.

While Wroth (1998) and Onfroy et al. (1999) found no evidence for *A. pinodes* pathotypes among Australian and French isolates based on virulence, Ali et al. (1978) reported that in Australia, 15 pathotypes existed for *A. pinodes*. Onfroy et al. (1999) screened 10 *A. pinodes* isolates on six field pea genotypes, and scored severity of infection on the first four leaves and internodes of inoculated plants grown under controlled conditions in a glasshouse. Ali et al. (1978) determined pathotypes based on the degree of lesion development on leaves and stems of 38 field pea genotypes grown in field trials. In Canada, Xue et al. (1998) differentiated 22 pathotypes of *A. pinodes* by their differential reaction on 21 field pea genotypes; pathotypes specific for leaf (16) and stem (9) infection have also been reported (Clulow et al. 1991). Ali et al. (1978) also reported the existence of 13 pathotypes for *A. pisi* using a similar assessment to that used for identifying pathotypes of *A. pinodes*. Differences in host or organ infection by different isolates may be related to differences in methodologies used in the studies and in the interpretation of the scoring for disease severity or aggressiveness. Qualitative differences in infection of genotypes by different isolates would give a clearer indication of the existence of pathotypes whereas quantitative differences could be interpreted as variation in aggressiveness within the population.

For *A. rabiei*, the classification of isolates from Syria into three pathogenicity groups (I, II, III) has been widely accepted (Udupa et al. 1998; Chen et al. 2004; Jayakumar et al. 2005). Udupa et al. (1998) found quantitative levels of infection (aggressiveness) among 53 isolates on three host genotypes. In Australia (Pradhan 2006), USA (Chen et al. 2004) and Canada (Jayakumar et al. 2005), pathotypes I and II have been recorded, while in India there were reports of up to 13 pathotypes (Vir and Grewal 1974), and 3 in Pakistan (Jamil et al. 2000). However, these reports of pathotypes were based on severity of infection on a small range of genotypes and were simply a measure of aggressiveness or virulence within the population of *A. rabiei* in each country. In Canada, Chongo et al. (2004) reported the presence of 14 pathotypes of *A. rabiei* based on quantitative differences in infection of stem and leaves of eight chickpea genotypes. Although a range of quantitative levels of infection were shown, only two isolates showed a qualitative difference in infection where they were unable to infect the resistant genotype ILC4421 compared to all the other isolates that infected this genotype. Thus these were the only two isolates that represented a second pathotype. The range of virulence or qualitative infection in ILC4421 for the other 38 isolates of pathotype 1 was from 0.4 to 2.4 on a 0–9 scale. The most aggressive isolate was able to infect all genotypes with the least level of infection occurring on the resistant genotypes FLIP83-48 and ILC4421.

Interestingly, in Israel Lichtenzveig et al. (2005) could not identify pathotypes although both mating types of the pathogen were detected in all chickpea-growing areas of the country. Israel is geographically close to the centre of origin of chickpeas and thus it may be assumed that co-evolution of *A. rabiei* and chickpea had occurred; however, none of the isolates screened belonged to the more aggressive pathogenic types II and III reported in Syria Udupa et al. (1998).

For *A. lentis*, six pathotypes were identified in Australia (Nasir and Bretag 1997). Although several of these pathotypes were associated with specific resistance genes in different lentil genotypes, the assessments were based on quantitative differences in pathogenicity. Since resistance to *A. lentis* was found

to be controlled by specific resistance genes (Ford et al. 1999; Nguyen et al. 2001), there is the likelihood that pathotypes of *A. lentis* evolved that had qualitative differences on lentil genotypes. In contrast, Banniza and Vandenberg (2006) reported that the host reaction of 16 lentil genotypes to 65 isolates of *A. lentis* collected in Canada resulted in a continuum of severity of infection. These results indicated natural variation of aggressiveness in the population without any distinct pathotypes. For *A. fabae*, physiological specialisation between host genotype and pathogen isolate has been proposed with identification of up to seven pathotypes (Rashid et al. 1991).

In order to better determine and compare the pathogenic diversity among isolates from different growing regions around the world, the adoption of an accepted set of host genotypes that are differential in their disease reaction to each of the particular *Ascochyta* species and a standard screening method for scoring disease severity are required.

Although there is debate on the existence of specific pathotypes of each of the *Ascochyta* spp. pathogens on their respective hosts there is no doubt that the level of aggressiveness of isolates is an important consideration in resistance breeding programmes. Genotypes with partial resistance, that result in lower levels of infection will ultimately reduce the inoculum potential in the field and limit or slow down an epidemic potential. Resistance to ascochyta blight in temperate legumes such as chickpea and field peas has been shown to be quantitative (Timmerman et al. 2002; Flandez-Galvez et al. 2003) thus making it difficult for pathotypes to evolve where the pathogen has specific avirulence genes. Nevertheless, there is a need to standardise the screening and evaluation methods used in bioassays for identifying both the level of resistance in the germplasm and the level of aggressiveness of the pathogen. The severity of infection on a range of genotypes is usually measured using a 0–9 non-parametric scale where 0 represents complete resistance and 9 a high level of susceptibility. However, a parametric scoring system or quantitative measure of severity and incidence of infection has also been used to measure the level of infection eg % leaf area infected and size of lesion relative to stem size (Flandez-Galvez et al. 2003; Lichtenzveig et al. 2002; Chongo et al. 2004; Tivoli et al. 2006). In assessing

resistance to *A. rabiei* in chickpea, Flandez-Galvez et al. (2003) adapted the linear stem index scale of Riahi et al. (1990) to measure the number of lesions and lesion length in relation to stem length, and identified maturity resistance in adult chickpea plants. Lichtenzveig et al. (2002) evaluated disease response in chickpeas using an assessment based on the transformed 'area under the disease progress curve' (AUDPC) and found that resistance to ascochyta blight was conditioned by a single quantitative trait locus with other minor loci contributing to resistance.

In conclusion, the development of efficient diagnostic techniques to detect latent infection of ascochyta blight pathogens in seeds and plant tissue, the understanding of population diversity, and identification of pathogenic variation will assist in the management of ascochyta blight diseases. The detection of latent infection will restrict the importation of more aggressive isolates into countries where cool season food legumes are grown or prevent the spread into areas where the pathogen does not exist. Efficient sampling and PCR-based techniques are currently only available for detecting ascochyta blight of chickpea in seed, and need to be developed for the other ascochyta blight diseases. Further studies are required into the population genetics of the ascochyta blight pathogens as this will lead to an understanding of how the pathogen is likely to adapt or evolve to changes in the environment, such as exposure to fungicides and resistant plant genotypes. In studying pathogenic variation there needs to be consistency in selection of differential host genotypes, screening conditions and disease evaluation techniques for each of the *Ascochyta* species. Knowledge of pathogen genomic variation, pathotype diversity and aggressiveness within populations of each of the ascochyta blight pathogens is critical to the success of breeding programmes to select for resistant genotypes.

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REVIEW PAPER

Resistance to ascochyta blights of cool season food legumes

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Abstract Ascochyta blights are the most important diseases of cool season food legumes (peas, lentils, chickpeas, and faba beans) and are found in nearly all production regions. Despite having the same common disease name, the pathogen species differ for each of the crops. These diseases cause serious yield losses under favourable cool and humid conditions. Planting resistant cultivars is often the first choice and most economical means in managing the diseases. Therefore breeding for resistance to ascochyta blights has been an important objective of many cool season food legume research programmes. Systematic screening of germplasm collections at international research centres and other national research programmes have identified useful resistance sources that have been used successfully to breed resistant or tolerant cultivars. Genetic studies have revealed inheritance patterns of the resistance genes. Genetic linkage analyses and QTL mapping have identified molecular markers that could be useful for marker-assisted selection and gene pyramiding. In general, research towards developing resistance to ascochyta blights in cool season food legume faces mainly two limitations: the lack of availability of efficient resistance sources and the lack of a good understanding of the variability of the pathogen populations. Research efforts to alleviate these limitations should be pursued. Given that modern technologies of marker development and genomics are available, further advances in deploying resistance to manage ascochyta blights in this group of legume crops will depend on concerted efforts in developing accurate screening procedures with adequate knowledge of pathogen variability and identification of additional sources of resistance.

Introduction

Peas (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and faba bean (*Vicia faba*) are important food crops throughout the world and are produced on nearly 25 million hectares with annual production approaching 40 million metric tons (FAOSTAT 2004). Total production ranges from over 20 million metric tons for pea to about 4 million metric tons for lentil (FAOSTAT 2004). These cool season food legumes are affected by a number of foliar and root diseases that cause wide spread damage and in severe cases cause complete crop

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loss. The most important foliar diseases worldwide are ascochyta blights. Although the diseases are collectively referred to as ascochyta blights due to similar symptoms, the pathogen species differ for each of the crops (Hernandez-Bello et al. 2006) and host specificity is necessary for disease development. The ascochyta blight complex of pea involves three pathogens, *Ascochyta pisi*, *Mycosphaerella pinodes*, and *Phoma medicaginis* var. *pinodella* (formerly *Ascochyta pinodella*). The disease is a complex because the three pathogens cause more or less similar symptoms and they frequently occur together. In the case of lentil, the crop is affected by *Ascochyta lentis* that causes leaf and stem spotting, leaf drop, stem lesions and seed lesions that result in serious reductions of yield and crop quality. Ascochyta blight of chickpea is caused by *Ascochyta rabiei* (*Didymella rabiei*) that causes severe symptoms on the leaves, stem breakage and die back, and often is cited as causing complete crop loss (Nene and Reddy 1987). Similar symptoms on faba bean incited by *Ascochyta fabae* cause yield losses and reduce seed quality.

Resistance to ascochyta blight in the cool season food legumes has been sought through germplasm exploration, collection, and systematic evaluation. Sources of partial resistance have been identified in all of the cool season food legumes and the currently available resistance is being used in breeding programmes designed to develop cultivars with improved resistance. Since there are some recent reviews on breeding methods, screening procedures, the ascochyta diseases and biology of the pathogens (Bretag et al. 2006; Pande et al. 2005; Tivoli et al. 2006; Torres et al. 2006; Ye et al. 2002), we will in this review mainly focus on the recent developments in understanding the genetics of host resistance for each of the major cool season food legumes and point out immediate needs in research that in our opinion will further advance deployment of resistance in managing ascochyta blight in cool season food legumes.

Peas

Ascochyta blight of pea is a disease complex caused by three pathogens: *Ascochyta pisi* which causes well-defined lesions (spots) on leaves, stems and pods; *Phoma medicaginis* var. *pinodella*, previously

Ascochyta pinodella, which causes lesions on leaves and stems, and foot rot; and *Mycosphaerella pinodes* which causes blight starting with small purple to black spots, enlarging and turning brown to black. The disease complex, its epidemiology, screening techniques and management were recently reviewed (Tivoli et al. 2006). Methods of screening for resistance have relied on field nurseries and natural infection by the pathogen through dispersal of airborne ascospores from debris of previously infected pea crops. In general no complete resistance to the ascochyta blight complex has been identified in peas; however good sources of partial resistance have been identified and are being used in breeding programmes (Tivoli et al. 2006). Screening of the USDA-ARS collection of pea germplasm was successful in identifying sources of partial resistance to *M. pinodes* in five accessions (PI 142441, PI 142442, PI 381132, PI 404221 and PI 413691); however, none of the accessions were more resistant than the cv. Radley. Other sources of resistance have been identified and include cv. Carneval (Tar'an et al. 2003) and accessions JI 97 and JI 1089 from the John Innes Institute Collection. Accessions of the wild pea species, *P. fulvum* have also shown some resistance to ascochyta blight.

A relationship between lodging traits and resistance to ascochyta blight was found (Banniza et al. 2005) indicating that stem structural components may have a role in resistance. Thus, upright plant habit with resistance to lodging appears to be an important factor in reducing disease severity.

Most of the genetic studies on pea resistance focused on resistance to *M. pinodes* possibly because it is the most destructive pathogen of the three involved. The genetics of resistance to ascochyta blight in peas indicate a multiple gene system with some dominance and additive genetic effects (Wroth 1999). Estimates of quantitative trait loci (QTL) for resistance to ascochyta blight have ranged from three (Tar'an et al. 2003) to 13 (Timmerman-Vaughan et al. 2004) (Table 1). The complex nature of resistance, as indicated by the number of QTL that have been estimated, presents a challenge to breeders and the prospect of making use of marker assisted selection for ascochyta blight resistance in peas.

Development of cultivars with improved resistance to the disease will depend on the use of germplasm with partial resistance to ascochyta blight with

Table 1 Quantitative trait loci (QTL) associated with ascochyta blight resistance in peas

Loci	% Variation accounted for	Reference
6 QTL in seedling stage	76.0	Prioul et al. (2004)
10 QTL in adult stage	56.6–67.1	Prioul et al. (2004)
14 QTL in 7 linkage groups	Not estimated	Timmerman-Vaughan et al. (2004)
3 QTL	35.9	Tar'an et al. (2003)

consideration for plant traits such as good standing ability that has been shown to be associated with resistance. In general, developing pea cultivars resistant to ascochyta blight is rather challenging because of the number of pathogens involved, pathogenic variation (races or pathotypes) within each pathogen species and seemingly tissue or growth stage specificity of certain resistance genes, in addition to lack of efficient resistance sources. Marker-assisted selection may be attempted but the number of QTL that are estimated to be involved with disease expression would seem to make that approach difficult and time-consuming. Direct screening in the presence of the disease may be a more viable approach at the present time until efficient marker-assisted selection protocols are established. Needless to say, more efficient resistance sources are needed and additional exploration and collection in regions of diversity may be a fruitful approach. Also, the use of wild species such as *P. fulvum* may hold promise as a source of resistance genes.

Lentil

Ascochyta blight of lentil, caused by *Ascochyta lentis* (teleomorph: *Didymella lentis*), has world-wide distribution and causes extensive damage to yields and crop quality. The disease causes necrotic spots on the leaflets, stems, pods and seeds. The lesion spots are initially light grey and turn tan, and are surrounded by darker margins. Lesions often enlarge and coalesce causing blight and stem breakage. Tivoli et al. (2006) provided a thorough review of sources of resistance and screening techniques for ascochyta blight of lentil and Ye et al. (2002) gave an account of breeding techniques for selection of lentils with resistance to ascochyta blight. Partial resistance to the disease is available in the germplasm. Most notable of the partially resistant germplasm accessions are lentil accessions PI 339283, PI 374118, ILL5588,

ILL5684, PR86-360, and ILL7537. Other accessions have been reported as resistant and have been used in breeding. The sources of resistance are readily available from gene banks at ICARDA, the U.S. Department of Agriculture-Agricultural Research Service, Canada and Australia. Screening for resistance has generally relied on field screening; however, screening in controlled environments has been practiced with good results (Muehlbauer, personal observations).

Resistance to ascochyta blight in lentil has been reported, but theories abound with the number and nature of genes responsible for the observed resistance. Two complementary dominant genes for resistance were postulated (Ahmad et al. 1997) in a cross of *L. ervoides* × *L. odemensis* whereas a single dominant gene was found in crosses within *L. culinaris*. Ford et al. (1999) identified a single dominant gene, *Abr*(1), in accession ILL5588 that conferred resistance to ascochyta blight in lentil and also identified molecular markers flanking the resistance gene that may be useful in marker-assisted selection. Chowdhury et al. (2001) postulated that a single recessive gene conferred resistance to ascochyta blight in lentil and was linked to RAPD markers, UBC227 and OPD-10. These RAPD markers are currently being used in marker-assisted selection. Nguyen et al. (2001) studied the resistance in germplasm accession ILL7537 and found that two complementary dominant genes conferred resistance. Ye et al. (2003) found two dominant genes in ILL5588 with one gene for resistance and the other for partial resistance, and one or two recessive genes in Laird and Indianhead, respectively. Additionally, two complementary resistance genes were found in the susceptible lines W6-3192 and Titore (Ye et al. 2003). At least five QTL for blight resistance have been mapped on four linkage groups and they together accounted for 50% of phenotypic variation (Rubeena et al. 2006). It appears that at least two genes are involved in resistance to ascochyta blight in

lentil, and the nature of the genes (whether dominant or recessive) depends on the sources. Based on current information it is not possible to make comparisons of the genes that have been identified or to draw conclusions on the number of genes involved. Appropriate allelism tests need to be conducted using common parents followed by uniform and systematic screening of the progenies. It is clear that the expression of those genes may be altered by variable environmental conditions which can alter the interpretation of the mode of action of the genes. Nevertheless, their use in breeding has led to the development of resistant cultivars such as Milestone (Vandenberg et al. 2001) and other candidate breeding lines with resistance.

Variation in virulence patterns of *Ascochyta lentis* has been reported (Ahmed et al. 1996; Ahmed and Morrall 1996; Nasir and Bretag 1997) and six pathotypes have been classified in Australia (Nasir and Bretag 1997). The cultivar Laird, released in Canada, was initially described as resistant to ascochyta blight but is now classified as susceptible. The reduced resistance of cv. Laird was reported to be due to the appearance of more virulent pathotypes (Ahmed et al. 1996). The pathogenic variation has undoubtedly contributed to the confusion about the genetics of resistance in lentil to ascochyta blight. Needless to say, these findings have important implications for lentil breeding and point out the need to consider pathogen variation during evaluation and selection for improved resistance.

Chickpea

Ascochyta blight, caused by *Ascochyta rabiei* [teleomorph: *Didymella rabiei* var. Arx], is responsible for widespread damage to chickpea crops worldwide. The disease causes necrotic lesions on the leaflets, stems, pods and seeds. Symptoms initially appear as water-soaked lesions on stems and leaves and turn to sunken, dark brown lesions with concentric black speckles of pycnidia. Lesions enlarge and coalesce under conditions favourable to the disease, causing leaf blight, stem girdling, stem breakage and plant death. Pande et al. (2005) provided a review of pathogen biology and the disease management of ascochyta blight. A thorough review of ascochyta blight of chickpea and available sources of resistant

germplasm was recently completed (Tivoli et al. 2006). Most notable of the partially resistant germplasm include accessions from ICARDA such as ILC-72, ILC-3279, ILC-3868, ILC-3870, ILC-3996 and numerous FLIP lines that have shown resistance at multiple locations (Reddy and Singh 1984). Breeding lines from ICARDA such as FLIP90-98C, FLIP91-22C, FLIP91-46C, FLIP91-2C, FLIP91-24C, FLIP91-50C, FLIP91-54C, and FLIP91-18C, developed from resistance sources ILC-72 and ILC-3279, have also shown a degree of resistance in the field and in controlled environments (Singh and Reddy 1994). These accessions and others developed at ICARDA have been used in breeding programmes worldwide to develop resistant cultivars. Progress continues to be made in the development of breeding lines with improved resistance to the disease.

Studies of the genetics of resistance to ascochyta blight have relied on the use of recombinant inbred lines (RILs) from crosses between resistant and susceptible parents and QTL analyses. Santra et al. (2000) used a set of RILs from the cross of FLIP84-92C × *C. reticulatum* (PI 599072) to identify two QTL (*QTL*-1 and *QTL*-2) that in combination accounted for 50.3 and 45.0% of the variation in blight scores, respectively, over two years of evaluation. Other studies (Flandez-Galvez et al. 2003; Collard et al. 2003; Iruela et al. 2006) have identified QTL for blight resistance in comparable regions of the genome as those found by Santra et al. (2000) providing confidence in the presumed locations of the resistance genes and prospects for marker-assisted selection and eventual map-based cloning. Likewise, Lichtenzveig et al. (2006) found three QTL for resistance that were located on comparable linkage groups, and a significant epistatic interaction of the resistance QTL on linkage group 8 with flowering time.

Marker density in the *QTL*-1 region of the chickpea genome was increased by Rakshit et al. (2003) who used bulked segregant analysis and DNA amplified fingerprinting (DAF) to identify a marker directly at the peak of *QTL*-1 of Santra et al. (2000). Millan et al. (2003) also identified additional markers linked to resistance and showed their potential use in selection. Efforts are currently underway towards fine mapping of *QTL*-1 using Bacterial Artificial Chromosome (BAC) libraries (Rajesh et al. 2004). The BACs of interest are being identified through the use

of markers associated with *QTL*-1 followed by BAC end sequencing to identify single nucleotide polymorphisms for conversion to CAPs and dCAPs markers. Those markers are then being used to increase marker density within *QTL*-1. The increased marker density provides additional markers for possible use in marker-assisted selection and should facilitate cloning and characterization of the resistance genes.

The existence of pathotypes of *Didymella rabiei* must be considered in breeding programmes designed to develop resistant cultivars. There has been a plethora of classification schemes for pathogenic variation in *D. rabiei*, ranging from an initial description of six races of the pathogen (Singh and Reddy 1993) to 14 virulence forms or pathotypes (Chen et al. 2004). The current trend is a more workable classification into either two or three pathotypes (Udupa et al. 1998; Chen et al. 2004). Using a mini-dome technique, Chen et al. (2004) was able to assign isolates of *A. rabiei* from the U.S. Pacific Northwest into two pathotypes (I&II). Isolates from the two-pathotype system were used to map pathotype-specific QTL conferring resistance and to study the mechanisms of resistance in the host (Chen et al. 2005; Cho et al. 2004; Cho et al. 2005). The two-pathotype system explains the evolution of US chickpea cultivars (Chen et al. 2004). The initial chickpea cultivars (such as Spanish White and UC-5) introduced into the US Pacific Northwest were shown to be highly susceptible to both pathotypes I and II; cultivars developed through breeding for resistance (such as Sanford and Dwelley) released in the early 1990s had only resistance to pathotype I, while more recently released cultivars (such as Sierra) were shown to have resistance to pathotype I and a high level of tolerance to pathotype II (Chen et al. 2005). Our current chickpea breeding efforts are to incorporate more efficient resistance genes to improve resistance against pathotype II and to avoid the potential emergence in the US of a new pathotype that is highly virulent on chickpea ICC 12004 reported in Syria (Bayaa et al. 2004).

Faba bean

Ascochyta blight of faba bean is caused by *Ascochyta fabae*, (teleomorph *Didymella fabae*) which is highly specific for faba bean. Lesions with definite margins are more or less circular or oval, slightly sunken on leaves, and more sunken on stems and pods. The disease, screening procedures and procedures for breeding disease-resistant faba bean cultivars were the subjects of a recent review (Tivoli et al. 2006). Screening for resistance has relied on the use of field nurseries and natural infection by the pathogen which may be supplemented by artificial inoculation with the pathogen or by the spreading of infected crop debris in the nursery area. Races of the pathogen have been suggested; however, classification into races has been controversial. Numerous sources of resistance listed by Tivoli et al. (2006) are being used in breeding programmes to develop improved cultivars.

A major dominant gene for resistance to ascochyta blight of faba bean was reportedly found in ILB752 and two complementary recessive genes for resistance were found in NEB463 (Kohpina et al. 2000). A detailed analysis of resistance using an F_2 population from the cross of 29H (resistant) × VF136 (susceptible) was used to identify six QTL (Avila et al. 2004). The F_2 population was evaluated for resistance to two isolates differing in their pathogenicity. Four of the QTL were effective against both pathotypes while the effectiveness of the two other QTL varied. Some QTL appeared to be tissue (either leaf or stem) specific (Avila et al. 2004), complicating selection protocols in breeding.

Variability of isolates of the ascochyta blight pathogen like those observed in Australia (Kohpina et al. 1999) is problematic for breeding and it is necessary to evaluate segregating breeding material against a range of isolates to ensure success.

Summary and conclusions

Ascochyta blights are an important yield constraint of all cool season food legumes, and using host resistance is the most economical means in managing the diseases. Resistance to ascochyta blights is present in the germplasm of all cool season food legumes; however, in most cases no complete resistance is found in the cultivated germplasm and the resistance is considered to be partial. Nevertheless, the available resistance has been demonstrated capable of reducing losses of yield and quality of these grain legumes. There is a pressing need for increased understanding

of pathogen variability, and for the standardization of screening procedures including the methods of inoculation and disease-scoring procedures, since the isolates being used for inoculation will be location-specific and disease progression will vary. Nevertheless, standardization of scoring procedures and the use of common host differentials and isolates as controls will enable comparisons of the data and results of evaluations across research locations.

The inheritance of resistance to ascochyta blights in cool season food legumes appears to be quantitative and controlled in most cases by multiple QTL. It is interesting to note that the number of QTL estimated using early generation populations such as F_2 is greater than the number of QTL estimated using nearly-homozygous recombinant inbred line populations, indicating that the latter may be a more realistic estimate of the inheritance of resistance and the location of the important genes. The use of marker-assisted selection for resistance to ascochyta blights is being developed in all of the cool season food legumes. However, it is still limited in scope, and its practical application requires further experimentation and confirmation. Selection under natural conditions in the field using a mixture of isolates remains the primary means of selection for resistance. The mini-dome procedure (Chen et al. 2005) has greatly improved the efficiency of evaluation of selections for resistance to multiple pathotypes in chickpea. Improved cultivars with resistance to ascochyta blights have been the result of breeding programmes worldwide. Seeking new resistance sources of additional germplasm lines or wild relatives will make it possible to continue to improve on that resistance. The prospect of pyramiding of genes, once identified, from various sources with the aid of modern molecular techniques has been discussed, and remains a possible fruitful approach for further improving resistance to ascochyta blights in cool season food legumes.

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