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REVIEW PAPER

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

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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*

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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

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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