# Determining frequencies of avirulent alleles in airborne inoculum of *Leptosphaeria maculans* using molecular assays

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## ABSTRACT

Control of blackleg (phoma stem canker) disease relies on crop management, fungicides and breeding for disease resistance. Proteins encoded by these resistance genes recognize avirulence (*Avr*) gene products in the fungus during invasion of the plant. However, these *Avr* genes can mutate resulting in fungal isolates becoming virulent, thus avoiding recognition by the plant. Widespread use of cultivars containing resistance genes provides strong selection for isolates that have mutated forms of *Avr* genes. This selection results in increased frequency of virulent isolates and accordingly, resistance genes become less effective. Molecular tools are now available to assess changes in frequencies of virulent isolates in populations of *L. maculans.* This paper describes the development of such tools for assessing changes in frequencies of virulent isolates within airborne (ascospore) pathogen inoculum.

**Key Words:** airborne inoculum – avirulence genes – *Avr* genes – *Leptosphaeria maculans*

## INTRODUCTION

*Leptosphaeria maculans*, the casual agent of blackleg (phoma stem canker) disease, is responsible for major yield losses of canola (oilseed rape, *Brassica napus*) worldwide. The interaction of *L. maculans* with canola at the seedling stage is a typical 'gene-for-gene' relationship. Ten *Avr* (*AvrLm*) genes that are involved in recognition of *L. maculans* by *Brassica* species have been identified (Balesdent et al. 2005; Van de Wouw et al. 2009) with three of them, *AvrLm1, AvrLm6* and *AvrLm4-7,* cloned (Gout et al. 2006; Fudal et al. 2007; Parlange et al. 2009).

*AvrLm1* and *AvrLm6* confer avirulence towards the resistance genes *Rlm1* and *Rlm6*, respectively (Gout et al. 2006; Fudal et al. 2007). Virulence towards these genes is primarily associated with deletion of the entire open reading frames of *AvrLm1* and *AvrLm6* (Gout et al. 2006; Fudal et al. 2007). Deletion of *AvrLm1* was responsible for virulence in over 90% of 460 virulent isolates genotyped from France, Mexico and Australia (Gout et al. 2007). Monitoring the frequency of avirulent and virulent isolates within fungal populations can play an important role in predicting and managing the risk of disease epidemics. Currently frequencies of avirulent alleles within fungal populations are determined by laboriously genotyping single isolates cultured from diseased plants at various locations each year. *Avr* genotypes are determined by pathogenicity testing on cultivars with corresponding resistance genes and/or using molecular markers for avirulent alleles.

Another potential method for estimating avirulent allele frequencies from fungal populations is molecular analysis of airborne spore samples (West et al. 2008). Spore-based forecasting systems are successfully predicting the occurrence and spread of diseases such as soya bean rust (*Phakospora pachyrhizi*) and cereal rusts (*Puccinia spp.*) in the USA, and blackleg on canola in Poland. Disease risk predictions are based on the timing of onset of spore release (an increase above a small background level) detected in airborne samples (West et al. 2008). However, these sampling methods do not provide data about changes in frequencies of particular genes, such as *Avr* genes.

The purpose of this study is to develop molecular techniques, specifically quantitative PCR, to determine frequencies of avirulent alleles in airborne inoculum. We will use these techniques to assess the changes in allele frequencies for *AvrLm1* and *AvrLm6* across three canola growing seasons in the UK.

## MATERIALS AND METHODS

Two methods are used to collect samples of airborne inoculum. Firstly, for initial testing of the method, artificial samples of airborne inoculum are generated by ejecting ascospores from individual pieces of diseased canola stubble. Pieces of wax-coated Melinex plastic tape (14 x 48 mm) are placed on microscope slides in an up-turned lid of a Petri dish. Pieces of diseased stubble of cv. NK Bravour (with *Rlm1*) are attached to the inside of Petri dishes using Vaseline and misted with sterile water to stimulate ejection of ascospores. After 30 to 60 min, the ascospore tape is removed and cut longitudinally into two subsections (each 7 x 48 mm). Using a light microscope, the number of ascospores is counted from the entire surface of each subsection of tape before it is transferred into 2 ml screw-top tubes for extraction of DNA (see below).

Secondly, field samples of airborne inoculum have been collected at Rothamsted Research, Harpenden, Hertfordshire, UK during the 2006/2007, 2007/2008 and 2008/2009 canola growing seasons using a Burkard seven day volumetric spore sampler. Stubble of diseased canola was located within 5 m of the Burkard spore sampler, which was operating according to standard methods described in Lacey and West (2006). Weekly strips of Melinex tape were divided into daily sections (14 x 48 mm) as described previously (Kaczmarek et al. 2008). These were then cut longitudinally, with one subsection used for extraction of DNA (see below) whilst the other was used to estimate the number of ascospores collected that day. This subsection was mounted on a microscope slide and the number of ascospores was determined visually by counting all ascospores of *L. maculans* in a similar way to that used for *Sclerotinia sclerotiorum* ascospores described in Rogers et al. (2009). DNA was analysed for proportion of avirulent alleles on dates when the visual counts showed that relatively large numbers of ascospores were present and on at least two different dates in each growing season.

DNA is extracted from either individual isolates grown in culture or from ascospores attached to subsections of Melinex tape using the CTAB extraction protocol (Rogers et al. 2009). DNA samples from isolates of known genotypes are mixed together in different proportions to generate a series of isolate mixtures that are used to assess whether quantitative PCR can discriminate different frequencies of avirulent alleles. These mixtures are created to represent populations with 100, 80, 60, 40, 20 or 0% frequencies of avirulent alleles.

When amplifying the *AvrLm1* and *AvrLm6* genes, production of a band following PCR represents the avirulent allele, whilst lack of amplification indicates the virulent allele. Primers specific for amplification of the *L. maculans* internal transcribed spacer (ITS) of ribosomal DNA are used as a positive control. In each quantitative PCR run a standard curve will be generated by plotting quantities of DNA against cycle threshold ($C_t$) values. The resulting regression equations will then used to quantify the amount of DNA in 'unknown' samples. Allele frequencies will be estimated by comparing the quantity of avirulent allele DNA with the quantity of ITS DNA amplified in each sample.

The genotypes of all cultured isolates are determined by assessing their ability to infect cultivars with known resistance genes (Van de Wouw et al. 2009). The cultivars used are Westar (no resistance genes), Darmor-MX (*Rlm6*) and Columbus (*Rlm1*, *Rlm3*).

## RESULTS

The ITS and *AvrLm6* regions were amplified from samples of artificial airborne inoculum using *L. maculans* specific primers. There was direct relationship ($R^2 = 0.79$) between quantity of ITS DNA amplified and number of ascospores in each sample (Fig. 1).

The frequency of the *AvrLm6* avirulent allele was estimated in each sample by comparing quantities of *AvrLm6* DNA with quantities of ITS DNA (Fig. 1). Frequencies ranged between 71 and 93% of the *AvrLm6* allele in all but two artificial airborne samples. In the remaining two samples, frequencies were greater than 100%. These two samples contained less than 50 ascospores, suggesting that this assay is inappropriate for spore samples with low ascospore numbers.

Frequencies of *AvrLm1* and *AvrLm6* alleles will be determined from field samples of airborne inoculum collected during 2006/2007. Also, 24 individual isolates collected at Rothamsted during the 2006/2007 growing season, will be genotyped by pathogenicity testing

and the frequency of *AvrLm1* and *AvrLm6* alleles determined. Allele frequencies of *AvrLm1* and *AvrLm6* estimated using both techniques will be compared to assess the accuracy of the molecular assay.

Lastly, frequencies of *AvrLm1* and *AvrLm6* avirulent alleles will be determined from field samples of airborne inoculum collected during three growing seasons, 2006/2007, 2007/2008 and 2008/2009.

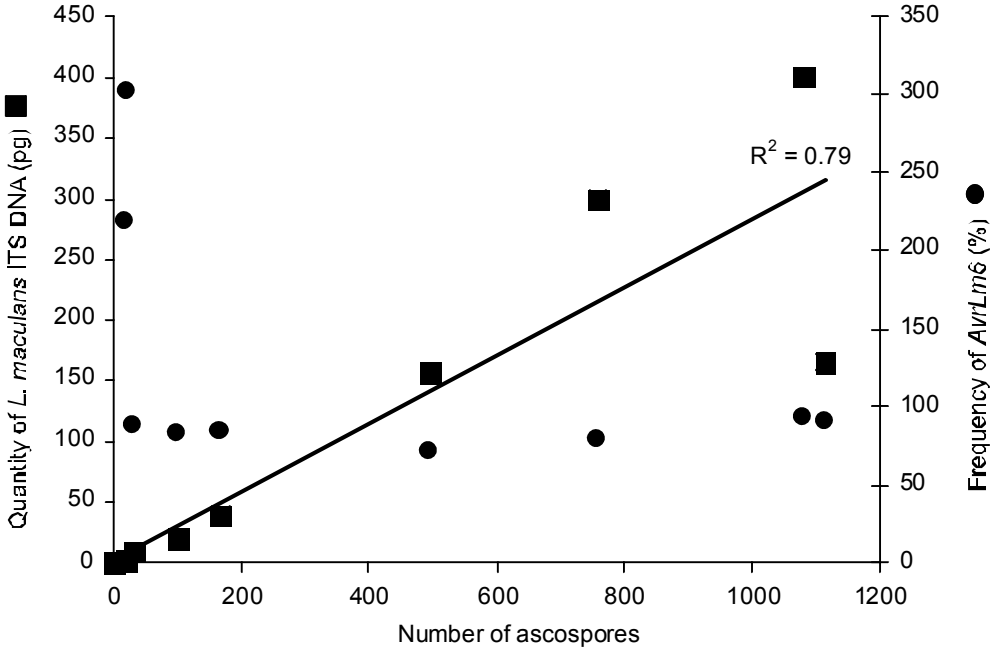


Fig. 1. Relationships between number of ascospores in artificial samples of airborne inoculum and quantities of DNA amplified with primers specific for the internal transcribed spacer (ITS) of the ribosomal DNA of *Leptosphaeria maculans* (■) and the estimated frequency of *AvrLm6* allele (●). The linear relationship between ascospore number and quantity of ITS DNA has been shown by regression analysis.

## DISCUSSION

Quantitative PCR is a rapid and accurate method for estimating avirulent allele frequencies in airborne *L. maculans* inoculum. This type of monitoring analyses extremely large populations of ascospores collected from multiple stubble sources. Although the sampled population will still be influenced by the types of cultivars being grown in fields adjacent to the trap, the samples will be less biased compared to estimating allele frequencies from small numbers of single isolates cultured from particular stubble sources. The accuracy of detection using airborne inoculum was limited by ascospore number. Therefore when applying this technology, only airborne inoculum samples with high quantities of ascospores should be analysed.

Application of the technologies developed by this study may allow monitoring of genetic changes in pathogen populations, including *Avr* genes. Additionally this technology may be applicable to other systems including monitoring the level of resistance to triazole fungicides, for example, if a mutation in a target gene has been identified.

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