Detecting Airborne Inoculum of Fungal Pathogens of Oilseed Rape Using Polymerase Chain Reaction (PCR) Assays

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ABSTRACT

Leptosphaeria maculans and *Pyrenopeziza brassicae* are damaging pathogens of oilseed rape that are spread by airborne ascospores. The use of DNA-based methods for detecting airborne inoculum of the two pathogens was investigated. A method for purifying DNA from spores collected using Burkard spore samplers (Burkard Manufacturing Co., Rickmansworth, UK) and detecting it using polymerase chain reaction (PCR) assays is described. For both pathogens the spore trap tape and the spore-collecting medium had little effect on the sensitivities of the assays compared with pure spore suspensions. As few as 10 spores of *L. maculans* or *P. brassicae* could be detected by PCR and spores of both species could be detected in a background of spores of six other species. The method successfully detected spores of *P. brassicae* collected using spore traps operated in oilseed rape crops that were infected with *P. brassicae. L. maculans* spores were detected using spore traps operated on open ground close to *L. maculans*-infected oilseed rape stems.

Key words: *Leptosphaeria maculans*, *Pyrenopeziza brassicae*, PCR, ascospores, inoculum detection.

INTRODUCTION

Leptosphaeria maculans and *Pyrenopeziza brassicae* are two of the most damaging pathogens of oilseed brassicas, particularly winter oilseed rape. *L. maculans* causes phoma stem canker, which can be economically damaging in many areas of the world, particularly in Europe, Australia and Canada. *P. brassicae* causes light leaf spot, one of the most damaging diseases of winter oilseed rape crops in the UK, and northern Europe. As no cultivars are available that are fully resistant to either pathogen disease control relies heavily on the use of fungicides. To make efficient use of disease control measures forecasting systems are needed to predict the risk of severe epidemics.

Both pathogens can be dispersed by airborne ascospores that are probably responsible for the initial infection of winter oilseed rape crops in the autumn. Thus the ability to monitor airborne inoculum would give a direct measure of when crops were vulnerable to infections. Conventional methods of monitoring airborne inoculum are time consuming, require experienced personnel and may be unreliable. Molecular techniques, based on DNA analysis, have been recognised as potential alternative methods. We report the results of experiments to determine the potential of combining air sampling, using Burkard spore traps, with polymerase chain reaction (PCR) assays to detect airborne inoculum of the two pathogens.

MATERIALS AND METHODS

Spore trapping and air sampling. Naturally released ascospores of *P. brassicae* and *L. maculans* were collected using Burkard 7-day recording spore traps. The traps collected airborne particles on wax-coated Melinex tape (Burkard Manufacturing Co., Rickmansworth, UK) attached to a slowly rotating drum, allowing the recording of particle concentration over a 7-day period. One trap was operated on open ground and surrounded by pieces of oilseed rape stems, showing symptoms of stem canker and actively releasing *L. maculans* ascospores. A second trap was operated in a winter oilseed rape crop (cultivar Apex) that showed symptoms of light leaf spot (*P. brassicae*). Each spore trap tape was cut into 24mm sections, representing 12h exposure periods. Each section was cut in half along its centreline. One half was

examined under a light microscope to determine whether or not *L. maculans* or *P. brassicae* spores were present and the other half was placed in a 0.5ml microtube for DNA extraction and PCR analysis (see below). Pieces of spore trap tape containing known numbers of *L. maculans* or *P. brassicae* spores were prepared from spore suspensions and these tapes were used to test the DNA-extraction/PCR protocol.

DNA purification from spore trap tapes. Spores were removed from Burkard spore trap tapes by milling tape samples in the presence of small glass beads (acid washed Ballotini beads, 8.5 grade, 400-455µm diameter) in a FastPrep® machine (Savant Instruments, Holbrook, New York, USA). This also disrupted the spores giving a suspension of spore contents. Details of the extraction process are given in Calderon *et al* (2002a, b). DNA was purified from this suspension using the method described by Williams *et al* (2001).

PCR detection of P. brassicae and L. maculans spores. Purified DNA from spore trap tapes was amplified in PCR-assays using either consensus fungal primers (ITS4 and ITS5, White *et al,* 1990) or primers specific to the target pathogen. Specific detection of *P. brassicae* was done using a nested PCR, with primer pair Pb1 and Pb2 (Foster *et al,* 1999) in the first round and PbN1 and PbN2 (Foster, 2002) in the second round. *L. maculans* DNA was detected using specific primers D1 and D2 (Taylor, 1993). Details of the primers and protocols are given by Calderon *et al,* (2002b).

RESULTS

DNA was successfully purified from spore suspensions of *P. brassicae* and *L. maculans* and detected using the PCR assays. For both fungi it was possible to detect the DNA equivalent of about 10 spores in the PCR assays. *P.* brassicae and *L. maculans* DNA was also detected in spore suspensions containing equal numbers of spores from five other fungal species as well as the target spores. This demonstrated that the protocols could detect target spore in the presence of large numbers of spores from other fungi. The PCR assays also detected *P. brassicae* and *L. maculans* DNA in samples extracted from Melinex tapes containing the target spores. The sensitivity was similar to that for spore suspensions, showing that the spore tape or adhesive had little effect on the DNA extraction process or the PCR assays.

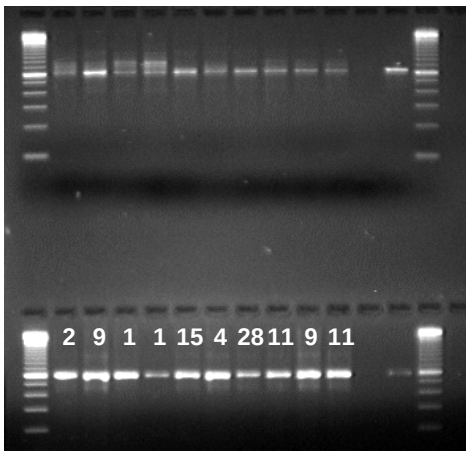

Figure 1. PCR assays of DNA prepared from field samples. The upper panel: consensus fungal primers and the lower panel: *L. maculans* specific primers. Lanes 1-10: DNA from 12-hour samples collected on selected days, numbers are spores estimated to have been in the PCR. Lane 11: no DNA. Lane 12: 50ng *L. maculans* DNA.

DNA was purified from 106 spore tape samples from the Burkard sampler that was surrounded by pieces of oilseed rape stem infected with *L. maculans* and from 194 samples from the sampler in the oilseed rape crop. DNA from all samples was amplified using the universal fungal rDNA primers ITS4/ ITS5. Several bands were amplified in individual samples, indicating the presence of several different fungal species (Figure 1). As expected a large number of different fungal spore types were found on the samples. Fifty-four of the 106 samples collected over *L. maculans*-infected oilseed rape stems were found to contain *L. maculans*-like spores, when examined under the microscope; *L. maculans* DNA was detected in 76% of these 54 samples. In the remaining 52 samples, no *L. maculans*-like spores were found and no *L. maculans* DNA detected. The number of *L. maculans* spores on 10 selected samples were

counted. For each of the 10 tapes analysed, *L. maculans*-like spores were observed on one half of the tape and *L. maculans* DNA was detected on the other (Figure 1). It was estimated that DNA from between 1 and 30 *L. maculans*-like spores was in the PCR, suggesting that the assay may have detected DNA from as little as one spore. One hundred and twenty-four of the 194 samples collected by the spore trap in the oilseed rape crop contained *P. brassicae*-like spores. *P. brassicae* DNA was detected on 44% of these, but not on samples where no *P. brassicae*-like spores were found. Because of the possibility of confusing *P. brassicae* ascospores with spores of other fungi we may have overestimated the number of *P. brassicae* ascospores. The number of *P. brassicae*-like spores on 13 tape samples was estimated and DNA was detected from four samples. No *P. brassicae* DNA was detected on the samples where no *P. brassicae*-like spores were found. DNA from between 14 and 25 *P. brassicae*-like spores was estimated to have been in the PCRs. The specific PCR assays detected the presence of *L. maculans* or *P. brassicae* spores in air-samples even where there was a high background of other spores and particles present.

DISCUSSION

PCR-based assays successfully detected the presence of inoculum of two pathogens of oilseed rape in air samples collected using a conventional spore trap. Using primers specific to the target pathogen relatively small numbers of spores could be detected, even in a background of many more non-target fungal spores. Several methods have been proposed, or used, for forecasting the risk of the development of epidemics of light leaf spot and stem canker. However, the ability to monitor the presence of airborne inoculum (ascospores) of both pathogens would potentially give a direct measure of when crops were vulnerable to infections. Unfortunately, the difficulty in monitoring airborne inoculum using conventional methods limits the practicality of such forecasting methods. The results presented here suggest that PCR-based assays have potential for routine monitoring of the presence of airborne inoculum of these two pathogens and could be developed as an additional tool for use in disease risk forecasting.

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