ACKNOWLEDGEMENTS

This work was supported by the Ministry of Agriculture, Fisheries and Food, Horticulture and Potatoes Division (HP0132). We also thank Hazel Davey, Dave Broadhurst, Helen Johnson, David Summers, Pat Causton, Peter Henley and John Parry (UW Aberystwyth); Tim Carver, Elwyn Jones and Ian Thomas (IGER), Nick Bradshaw and Aldwyn Clarke (ADAS), Dale Travis and others at Dycor, and Carsten Herrmann, Volker Ost and others at Partec GmbH .

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Use of flow cytometry in the detection of plant pathogenic spores

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ABSTRACT

A Partec PAS-III flow cytometer was used to differentiate sporangia of the late-blight pathogen *Phytophthora infestans* from other airborne particles. Using the PAS-III, light scatter and intrinsic fluorescence parameters could be used to differentiate sporangia from conidia of *Alternaria* or *Botrytis*, rust urediniospores and various pollens. Clear differentiation between *P. infestans* sporangia and *Blumeria* conidia was only possible using data analysis rules evolved using the methods of genetic programming, following staining with the fluorescent brightener Calcofluor white. Initial field data are presented and the potential application of these techniques to the prediction of late-blight epiphytotics in the field is discussed.

INTRODUCTION

Current methods for predicting the occurrence of late-blight of potato (*Phytophthora infestans*) rely on climatic modelling to identify conditions conducive to pathogen reproduction and thus to disease spread. Such methods can provide considerable savings to growers, as well as environmental benefits. In the UK, 'calendar spraying' is normal practice, with sprays commencing when the crop canopy has closed and continuing at 7-10 day intervals until crop desiccation / harvest. Use of forecasting models can reduce the number of required sprays by allowing a delay in the first fungicide application, and/or an extension of the interval between subsequent sprays (Hinds, 2000). However, such methods are not always reliable in all conditions. A multi-site evaluation of five forecasting systems from 1994 to 1997 indicated that their predictions differed from one another, and that the effectiveness of all the models varied from year to year. They were least effective in a 'low risk' year, when they recommended spraying even though no blight occurred (Bugiani *et al.*, 1998).

One important factor that is not taken into account by forecasting models is the amount of inoculum present in the immediate environment of the crop. Use of volumetric spore traps within potato fields, with identification and enumeration of sporangia carried out under the microscope, has given variable results. Schlenzig *et al.* (1998) detected inoculum in the air only after the first diseased plants were visible in the field, and concluded that sampling of the air was no more effective than a visual check of the crop in terms of identifying the start of the epidemic. In contrast, Bugiani *et al.* (1998) found that one or two 'peaks' in sporangia concentration in the air (10-20 sporangia m^{-3}) preceded the first observed symptoms, and used these data to confirm the predictions of climatic modelling. It is evident that the sensitivity of such methods would be greatly enhanced if an increased rate of sampling could be coupled with a means of identifying and enumerating the *P. infestans* sporangia automatically.

Flow cytometry is a well-proven technique that allows cells, spores and other particulates to be analysed individually (Davey & Kell, 1996; Shapiro, 1995). Particles are interrogated optically and, for each particle, measurements of several cellular parameters are recorded: these normally include forward light scatter, orthogonal ('side') light scatter, and one or more fluorescence parameters. Particles may show intrinsic 'autofluorescence' which may facilitates differentiation of

British Crop Protection Conference Brighton
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different species using flow cytometry (Vives-Rego *et al.*, 2000). In other cases, fluorescent dyes are needed to differentiate different microbes (Davey & Kell, 1996; Porter *et al.*, 1997). For example, forward and wide-angle light scatter coupled with DNA-binding fluorescent dyes have been used to differentiate spores of basidiomycete fungi (Allman, 1992).

Advances in computing and laser technology have recently led to the development of cheaper portable devices which can operate from a battery. Such devices offer the possibility of developing a field-based system, which if linked to a high-volume air sampler, could provide online measurement of airborne particles. The generic nature of the technology would allow the devices to be adapted to detect a wide range of plant pathogen propagules and thus provide advanced warning of impending epiphytotics. Here and in an earlier publication (Day *et al.*, 2002) we describe the use of flow cytometry linked to a high volume air sampler to provide sensitive and specific detection of *P. infestans* sporangia using a combination of staining with the fluorescent brightener Calcofluor white and genetic programming for data analysis.

MATERIALS AND METHODS

Source & preparation of fungal spores and pollen grains. An A1 mating type isolate of *P. infestans* (96.69) was maintained on rye A agar and sporangial suspension from plates were used to inoculate detached potato leaves. After 6-7 days incubation at 18°C, sporangia were washed from leaves with distilled water. Cultures of *Botrytis cinerea*, *Alternaria alternata* and *Penicillium chrysogenum* were maintained on potato dextrose agar and conidia were harvested by washing plates with distilled water. Conidia of *Blumeria graminis* var. *hordei* and rust urediniospores (*Puccinia coronata* f. sp. *avenae*, and, *P. recondita* f. sp. *tritici*) were harvested from infected plants by agitating leaves in 50 ml of distilled water containing a single drop of Tween-80. Grass, plantain and tree pollens were obtained locally. Spore / pollen suspensions were filtered through a single layer of muslin (average pore size ca. 0.5 mm) before being placed in the flow cytometer. For most analyses, particles were at a concentration of approximately 10^5 ml^{-1}. For some experiments, particles were fixed/killed by the addition of ethanol to the medium (50% final volume).

Staining of spores. A range of dyes were tested: $DiIC_1(5)$, $DiSC_3(5)$, TO-PRO, and SYTO (17, 59, 60, 61, 62, 63 and 64), Nile Blue A, FUN-1 and Calcofluor white M2R (Fluorescent Brightener 28; Tinopal UNPA-GX). Stock solutions were dissolved in dimethyl sulphoxide (DMSO) except for Nile Blue and Calcofluor white, which were made up in distilled water. After addition of dye, samples were routinely kept in the dark for 15 min at room temperature before being measured in the flow cytometer.

Flow cytometry. The Partec PAS-III Particle Analyzing System (Partec GmbH, Münster, Germany) was equipped with 488 nm argon-ion laser and 633 nm helium-neon lasers and a 100 W Hg arc lamp, and photomultiplier tube detectors for six optical parameters. For most experiments data were collected with respect to forward light scatter (FSC), side scatter (SSC), and four fluorescence (FL) parameters: FL1 (green; 515-560 nm), FL2 (orange; 575-605 nm), FL3 (red; >645 nm) and FL4 (red; 665-690 nm). For experiments using Calcofluor white, the PAS-III was operated with the 488 nm argon-ion laser and the mercury arc lamp as a UV light source. In this configuration, fluorescence from the UV excitation was recorded (at 450-460 nm) instead of SSC. Standard reference beads (6.0, 10.5, 41.5 and 66.0 µm diameter; AlignFlow Plus Flow Cytometry) were used for calibration. Data analysis was carried out using techniques of multiple gating (FCS Express, De Novo Software, Canada) and genetic programming (Gmax-Bio; Aber Genomic Computing,).

Air sampling. An XMX/2AL liquid impinger air sampler (Dycor Technologies Ltd., Edmonton, Canada) was customised to collect particles of 20 to 50 µm and coupled with a liquid impingement module to allow collection of particles into a small volume of water. A mesh screen over the inlet served to prevent very large particles from entering the system and particles smaller than 7 µm would be exhausted from the unit. A cowl and wind vane were added to permit air to be collected while facing into the prevailing wind. Air samples were collected by high-velocity impingement into 5 ml of distilled water in 50 ml disposable plastic centrifuge tubes with 1 mm nylon mesh, positioned above the liquid to reduce particle entrainment and loss of sample fluid by splashing. The flow rate on the XMX is variable and setting 5 (giving a measured flow rate of 600 $l.min^{-1}$) was found to be optimal, since some damaged sporangia were observed at higher settings.

RESULTS

Light scatter detection of *P. infestans* sporangia using the PAS-III. Peak channel numbers (PCN) for both FSC (forward scatter) and SSC (orthogonal or 'side' scatter) were plotted against particle width, particle length and particle volume, and against their logarithms. R^2 values >0.9 were obtained for FSC plotted against width, log(width) and log(volume) for the 'standard' beads but no such correlations were observed for the biological particles. Viable *P. infestans* sporangia and zoospores, *Blumeria* and *Botrytis* conidia and grass pollen showed relatively high FSC for their size (relative to calibration beads), whereas rust urediniospores, *Alternaria* conidia, killed *P. infestans* sporangia and *Plantago* pollen showed relatively low FSC. Correlation of side scatter with particle size was generally similar to, or better than, that of FSC (Day *et al.*, 2002). The ratio of SSC/FSC for killed sporangia and for grass / plantain pollens was relatively high compared to that of the opther fungal particles tested (fig. 1). It is also clear from fig. 1 that *Blumeria* conidia cannot be differentiated from sporangia of *P. infestans* using FSC and SSC alone.

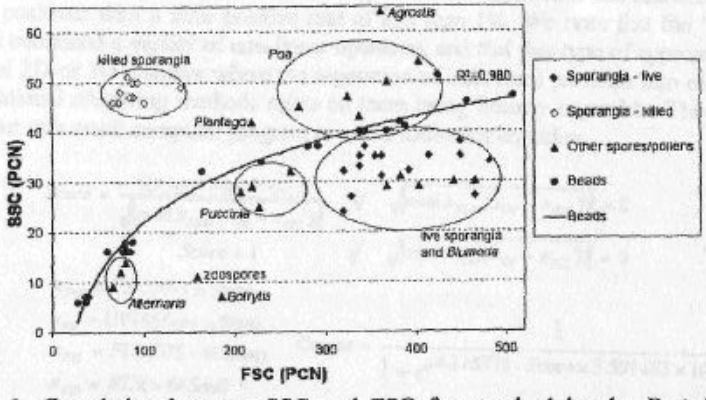

Figure 1. Correlation between SSC and FSC for standard beads, *P. infestans* sporangia and zoospores, other fungal spores and pollen. Logarithmic least squares best fit line calculated for beads only; R^2 calculated in MS Excel calculated using a transformed regression model.

Intrinsic fluorescence. Certain biological particles exhibited red 'autofluorescence', notably pollen, rust urediniospores, *Blumeria* conidia (particularly non-viable conidia), and, to a lesser degree, *P. infestans* sporangia. Both the fluorescence signals and the SSC signals are collected at an angle orthogonal both to the direction of the laser and to that of the sample fluid flow. Fluorescence signals

for each of the four wavelength 'windows' collected were plotted against SSC to determine which exhibited intrinsic fluorescence. Pollen, rust urediniospores, killed sporangia of *P. infestans*, and, to a lesser degree, viable *P. infestans* sporangia and *Blumeria* conidia showed varying degrees of orange and red FL (Fig. 2), which could be used as a further aid to differentiation.

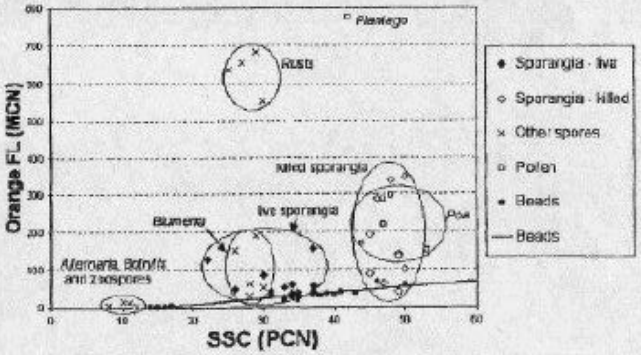

Figure 2. Correlation between orange autofluorescence (575-605 nm) and side scatter (SSC). *Agrostis* pollen also could not be shown on the same scale (Orange FL MCN > 3500). MCN = median channel number. Linear least squares best fit line for beads is shown.

Differentiation of *P. infestans* from other fungal spores using light scatter and autofluorescence. Using a combination of FSC, SSC and measurements of autofluorescence, *P. infestans* sporangia could readily be differentiated from all of the tested biological particles except for *Blumeria* conidia. Multiple gating was used to exclude particles that did not conform to the same FSC, SSC and FL parameters as the sporangia. For most of the spores (*Alternaria, Botrytis*, rusts) and pollens (*Agrostis, Plantago* and *Poa*) tested, <3% false positives were observed, though for *Blumeria* conidia, the number of false positives was extremely variable, ranging from 2% to 61% on different experimental days. Use of all six parameters for gating (i.e. inclusion of the two red FL parameters) did not consistently decrease the number of false positives, whilst always decreasing the number of true positives (i.e. the number of sporangia actually identified as sporangia).

Evaluation of fluorescent dyes. Sporangia and zoospores of *P. infestans* stained readily with nuclear dyes (TO-PRO-3®, SYTO® dyes), with the lipid-staining dye Nile Blue, with the membrane energization dyes $DiSC_3(5)$ and $DiI_1C(5)$ and with the fungal-specific stain FUN-1. These dyes were stained spores of *Penicillium, Alternaria* and *Botrytis* rather poorly, though they were less effective in differentiating sporangia from the various pollens tested. Differentiation of sporangia from pollen (but not from fungal spores) was more effective with SYTO-17 or TO-PRO-3, or with low concentrations (100 nM) of $DiSC_3(5)$. The multiple gating analysis was repeated for sporangia and other spores/pollens stained with these dyes, at concentrations of 1 µM or 10 µM. However, the number of false positives for pollens, *Blumeria* and rusts was either similar to or greater than the number that had been obtained in the absence of fluorescent dye. Thus, detection of *P. infestans* sporangia was not usefully improved by any of these red dyes.

The fluorescent brightener Calcofluor white stained sporangia and pollen effectively at a range of concentrations (10 µM, 100 µM and 1 mM), though sporangia were most effectively stained at 1mM. Staining of other fungal spores, including *Blumeria*, was much less effective. Multiple gating using FSC, UV and green and orange FL parameters indicated that Calcofluor

was effective for distinguishing sporangia from any of the other fungal spores tested (including *Blumeria*), and also from *Poa* pollen (Table 1).

Table.1. Percentage particles falling within gates defined for *P. infestans* sporangia, when stained with different concentrations of Calcofluor. Logical gating was used, where R1 and R2 were polygons drawn from plots of FSC vs green FL and UV vs orange FL respectively.

	Calcofluor concentration			
	0 µM	**10 µM**	**100 µM**	**1 mM**
P. infestans sporangia	58, 62, 67, 78, 87, 90, 90, 92, 92, 93, 96, 97, 97, 98, 98	69, 71, 77, 80, 86, 87, 89, 89, 90, 91, 92, 92, 94, 95	62, 77, 79, 81, 84, 85, 85, 85, 91	36, 55, 67, 72
Other fungal spores				
Alternaria	0	0	0	0
Blumeria	5, 31, 47, 65, 72	0, 0, 0, 0, 0	0, 1, 2	0
Brown rust	0, 0	0	0, 0	NT
Crown rust	0, 2	0	0, 0	NT
Pollens				
Anthoxanthum	0, 0	0, 0	0, 0	NT
Dactylis glomerata	1	0	NT	NT
Poa annua	0, 0, 1	0, 0, 3	0, 0, 1	0, 4, 5
Poa trivialis	10	0	NT	NT
Plantago	0, 0, 4	0, 0, 0	0	NT
Silver birch	1, 11	0, 1	NT	NT
Sycamore	0	0	0	NT

Use of Genetic Programming (GP) in Data Analysis. GP (Koza *et al.*, 1999) is a technique that allows the evolution of simple, interpretable rules from complex datasets. We have been applying GPs to a wide variety of problems in spectroscopy (Gilbert *et al.*, 1997; Johnson *et al.*, 2000). GPs proved to be a still more effective means of particle discrimination and allowed detection of 95% of true positives with a false positive rate of less than 1%. We note that the 'top' rule that was evolved contained a variety of non-linear operators, and that this type of approach is in contrast to the usual 2D or 3D displays where the separation of individual particles into classes via visual or computational clustering methods relies on them being linearly separable. This 'top rule' can be cast either as a small computer program or as the following equation:

$$Score = \frac{((x_{FL2} \times x_{UV}) - x_{FL1}^{2})}{\sqrt{\left|\min(x_{FL4}, (x_{UV} - x_{FL2}))\right|}} \quad if \quad \sqrt{\left|\min(x_{FL4}, (x_{UV} - x_{FL2}))\right|} \neq 0$$

$$Score = 1 \quad if \quad \sqrt{\left|\min(x_{FL4}, (x_{UV} - x_{FL2}))\right|} = 0$$

$$x_{FSC} = FSC(488 \pm 5nm)$$
$$x_{UV} = UV(455nm \pm 5nm)$$
$$x_{FL2} = FL2(575 - 605nm)$$
$$x_{FL3} = FL3(> 645nm)$$
$$x_{FL4} = FL4(665 - 690nm)$$

$$Output = \frac{1}{1 + e^{(4.118731 - Score \times 3.591183 \times 10^{-3})}}$$

Detection of *P. infestans* in samples of air spora spiked with sporangia. Samples collected in aqueous suspension using the XMX air sampler (on the UWA campus) on various dates during early 2001 were spiked using sporangia at 50–1000 ml^{-1}. Samples were counted in the PAS-III and the resulting data were analysed either using multiple gating or the equation derived from the GP. Good correlation was found between observed and expected values for sporangial numbers when samples were analysed using four-parameter multiple gating and even better with the GP equation.

Detection of *P. infestans* sporangia under field conditions. Preliminary experiments at the ADAS experimental site at Llanilar in 1999 and 2000 showed that sporangia were detectable above potato fields and that these could be trapped using the XMX sampler. Ten minute sampling periods were found to be optimal. Restrictions imposed following the Foot and Mouth outbreak rendered it impossible to carry out field testing at Llanilar in 2001. However, a 0.5 ha field on UWA property was planted with potato cultivars Rocket and Premiere. Samples from the air above the crop were collected on a weekly basis using the XMX and were analysed in the PAS-III (using both GP and multiple gating), as well as being examined microscopically (haemocytometer). For the GP using the equation described above, sporangia were counted as those particles with a probability 'score' above 0.80). The crop was also examined weekly for signs of late-blight, which was first observed (as a result of natural infection) in two foci on the first of August. Given that it would take 5 to 7 days for symptoms to become visible, and that the foci observed each involved several plants, we would estimate that initial infection probably occurred two weeks earlier.

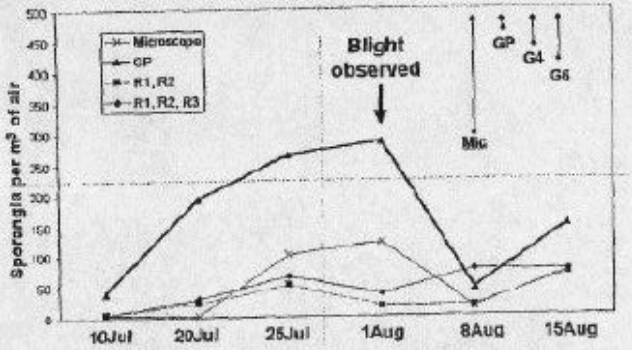

Figure 3. Estimated numbers of *P. infestans* sporangia m^{-3} of air, sampled above a potato crop planted at Penglais, Aberystwyth, in 2001 (mean of 3 samples per day). Vertical bars show LSD calculated by Duncan's Multiple Range Test of data from microscopic analysis (Mic), genetic programming (GP), and multiple gating using either four (G4) or six (G6) parameters. Significant differences in numbers of sporangia over the six sampling weeks were only indicated for data obtained by microscope ($P<0.001$) and GP ($P<0.05$).

Examination of the different methods of data analysis suggests that genetic programming (GP) was more effective than multiple gating (fig. 3). In each case, the variation between different samples taken on the same day was large, but in the case of the multiple gating data, such variation was so great that no significant difference was recorded between estimated sporangial numbers at the beginning, middle or end of the epidemic. Differences over time were observed, however, for the microscope data and the data analysed by GP. It is also apparent that the shapes of the graph are similar, for the microscope and the GP data, even though the estimated numbers of sporangia were lower for the microscope data. This may reflect the difficulty of counting sporangia in very dilute solutions under the microscope. The numbers of sporangia detected at different points in the epidemic (by GP) are higher than those reported from other studies, but not unreasonably so, e.g. over 60 m^{-3} from an infected crop (Gregory & Hirst, 1957). Bugiani *et al.* (1998) detected 'spikes' of around 20 sporangia m^{-3} prior to recording of disease occurrence, with 85 sporangia m^{-3} measured at the height of the disease progress curve. Numbers of pollen grains present in the samples were also estimated (up to 500 m^{-3}). Some attempts were made to differentiate pollen from different species,

DISCUSSION

A broadly linear relationship between log particle size and log FSC was observed for the 'standard' beads. This is in agreement with other experiments using either beads or physiologically-similar cells (Davey & Kell, 1996; Sharpless *et al.*, 1977)). As well as particle size, FSC is also influenced by refractive index (relative to the surrounding medium), by the presence in cells, or on their surface, of compounds that may absorb light at the illumination wavelength used, or by highly textured surface or internal structures which may also act to decrease the intensity of FSC (Sharpless *et al.*, 1977). These latter features have a greater influence on SSC than FSC, with SSC being less dependent upon particle size; typically, highest-intensity SSC signals are obtained from particles with the highest degree of cytoplasmic granularity (Davey & Kell, 1996). As well as the (fairly small) differences in FSC and SSC, differentiation of pollen and urediniospores from sporangia was facilitated by the intrinsic orange and red fluorescence of the former. Using a combination of FSC, SSC and FL parameters, sporangia could be distinguished from other fungal spores (except *Blumeria*) and pollen by multiple gating.

The ability of fluorescent dyes to enhance particle differentiation, in particular, to allow differentiation of *P. infestans* sporangia from *Blumeria* conidia, was assessed for a variety of different 'red' dyes. It was thought likely that fluorescent dyes would stain these two spore types differently, since *Blumeria* is a true fungus with chitin / glucan cell walls, while *P. infestans* is an oomycete classified in the Kingdom Chromista, with cellulose cell walls. However, for all the 'red' dyes tested, it appeared that the thickness or pigmentation of the wall influenced the effectiveness of staining more than did wall composition. The pattern of staining for *Blumeria* and pollen was similar to that for *P. infestans* sporangia. Moreover, the 'red' dyes tended to mask differences in intrinsic fluorescence, and so tended to make differentiation of sporangia from pollen and *Blumeria* less effective than for unstained particles.

Sporangia stained with Calcofluor could readily be differentiated using multiple gating from any of the other fungal spores tested, including *Blumeria*, with <1% false positives. Differentiation of sporangia from pollen grains using Calcofluor was less clear, since pollen stained in a similar manner to the sporangia. However, use of GPs allowed differentiation of sporangia from pollen, or from any of the other particles tested, again with <1% false positives at the level of the individual particle. Use of GP to analyse flow cytometry data could theoretically permit detection of a single sporangium within a complex sample. In practice, however, the detection threshold must be set higher in order to eliminate false positives. For example, if an 'early warning' system were set to trigger a warning for blight at, say, five sporangia per cubic metre of air, this would mean in theory that there could be up to 500 pollen grains present per cubic metre without triggering a warning (assuming <1% false positives for pollen).

Using GP, a clear increase in sporangial numbers was first apparent by 20th July, almost 2 weeks before symptoms were noticed in the crop. The system therefore appears promising, as a means of early blight detection. We recognise that more frequent sampling (including different years and at different sites) would be necessary for complete validation of the system. Differences in weather conditions are likely to affect the numbers of sporangia detected on different days. Indeed, even samples taken within minutes of each other demonstrated a high degree of variation in numbers of sporangia detected, suggesting that airborne spores travel in 'clouds', or, in the case of samples taken at the height of the epidemic, were liberated from the crop in irregular bursts, perhaps following gusts of wind. Sampling would certainly need to be carried out on a daily basis, with several samples taken per day.