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Rapid determination of *Phytophthora infestans* sporangia using a surface plasmon resonance immunosensor

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Abstract

Phytophthora infestans is the cause of late blight disease in potato and is an economically important pathogen worldwide. Early disease detection is important to implement disease control measures. In this study a surface plasmon resonance (SPR) immunosensor for detection of *P. infestans* sporangia is presented. The specificity of an existing mouse monoclonal antibody (phyt/G1470 mAb) against *P. infestans* was investigated in plate-trapped antigen ELISA and in subtractive inhibition ELISA. No or only limited cross-reactivity was observed against representatives having air-borne spores from *Ascomycetes*, *Deuteromycetes* as well as *Basidiomycetes*. phyt/G1470 mAb was incorporated in a subtractive inhibition SPR assay, consisting of a pre-incubation of mAb and sporangia, a centrifugation step to remove sporangia-bound phyt/G1470 mAb and quantification of remaining phyt/G1470 mAb by SPR. Good intra- and interday assay variability was observed and the assay had a detection limit of 2.2×10^6 sporangia/ml. Analysis time was 75 min, which is superior to existing *P. infestans* detection methods.

Keywords: Immunosensor; Plant disease; Monoclonal antibody; Surface plasmon resonance; *Phytophthora infestans*; Protein–protein interaction

1. Introduction

The fungus-like organism *Phytophthora infestans* is a major plant pathogen and the cause of late blight disease in potato and tomato plants in many parts of the world. *P. infestans* spreads by wind-dispersed sporangia produced on branched hyphae that emerge from the stomata of infected leaves under humid conditions (Judelson, 1997). Climate model decision support systems are used to predict favourable conditions for pathogen reproduction and disease spread (Bouma and Hansen, 1999). These models help farm managers in their decision to apply fungicide in a pre-emptive and rational manner. However, differences in climate and local *P. infestans* populations suggest that these methods are not always useful in geographical regions other than the one for which they were developed (De Visser and Meier, 2000; Hijmans et al., 2000; Kleinhenz and Jörg, 2000). Therefore there is an increasing demand for systems that can further aid in the early warning against *P. infestans*, such as direct detection of air-borne *P. infestans* sporangia.

For research and survey purposes, spore traps are typically used to collect air-borne *P. infestans* sporangia 'on-site'. Pathogens in the collected samples are identified by direct microscopic identification or by *in vitro* cultivation of spores and identification of characteristic fungal structures. This method is labour intensive, the throughput is low and analysis time can vary from days to weeks (Ward et al., 2004). Current molecular detection methods for *P. infestans* are based on polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA) (Bailey et al., 2002; Gough et al., 1999; Ristaino et al., 1998; Tooley et al., 1997). Although these assays are very sensitive and specific, they are labour intensive, label-dependent and therefore confined to a specialised laboratory environment. An alternative flow cytometry based assay which was able to discriminate *P. infestans* from other air-borne particles was recently described. The success of this approach was however dependent of sporangia labelling with fluorescent brightener and on complex data evaluation (Day et al., 2002). Future on-site detection sensors should preferably monitor air samples in a fast and continuous manner. This means that the

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detection should be label-free to minimise analyses time and should produce a result which can be interpreted by personnel with limited training.

In recent years there has been a focus on development of immunosensors for detection of food- and water-borne pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella* sp. and *Escherichia coli* (Bokken et al., 2003; Fratamico et al., 1998; Leonard et al., 2003; Vaughan et al., 2001) as well as various biowarfare pathogens (Deisingh and Thompson, 2004; Iqbal et al., 2000; Tims and Lim, 2004). Although detection of microorganisms in agriculture is highly important in order to limit crop losses, there is a lack of immunosensors for detection of plant pathogens.

Several different sensor types exist that can all be used as immunosensors, including Quartz crystal microbalance (QCM), cantilever-based sensors and surface plasmon resonance (SPR) (see reviews by Hansen and Thundat, 2005; Hepel, 1999; Karlsson, 2004; Marx, 2003). These sensors are capable of measuring biomolecular-interactions and display results in real-time. SPR-technology is the most widely used sensor type and the optical detection principle is used in many commercial sensors. In SPR systems it is possible to study 'real-time' molecular interactions and the technology has been used for detection of several different analytes (for a review see Karlsson, 2004). The most widely used SPR sensor is the Biacore® system, in which the binding of analytes to immobilised ligands can be studied. The sensor measures refractive index changes on and within 300 nm from the sensor surface, and the refractive index changes are proportional to the mass changes that occur upon analyte binding. Biacore® immunosensors have been used for the detection of bacterial cells, either by their binding to immobilised antibodies on sensor chip surfaces or by indirect subtractive inhibition assays (Bokken et al., 2003; Fratamico et al., 1998; Haines and Patel, 1995; Leonard et al., 2004). As the diameter of most cells exceeds the 300 nm range, a large number of cells are needed to produce a significant change in response units (RU) in assays with direct surface-capture (Hearty et al., 2006; Leonard et al., 2004; Oli et al., 2006; Quinn et al., 1997; Quinn et al., 2000).

The subtractive inhibition assay is an indirect method and is based on a pre-incubation of cells and antibody, removal of cell-bound antibody, followed by a quantification of the remaining unbound antibody by a Biacore® sensor surface capable of antibody binding. The remaining free antibody is thereby related to the initial cell concentration (Haines and Patel, 1995; Leonard et al., 2004). This makes detection of large cells/spores both easier and more sensitive compared to direct capture assays as the assay is reduced to a protein–protein interaction (Haines and Patel, 1995; Leonard et al., 2004). Furthermore, the method is good for analyses of complex matrices such as food samples, homogenised plant tissue, viscous material and dirt samples, which can all potentially block the microfluidic path and damage the sensor instrument (Bergwerff and van Knapen, 2006).

An SPR immunosensor capable of detecting *P. infestans* could be a useful supplement to early warning climate models. The wind-dispersed *P. infestans* sporangia are large, with a diameter range of 12–23 μm and could therefore potentially block the microfluidic path of a Biacore® sensor, thus making a direct sporangia capture SPR assay unsuitable. In this study an existing *P. infestans* mouse monoclonal antibody (mAb) is used in conjunction with a subtractive inhibition assay approach to develop a label-free SPR immunosensor for sporangia detection.

2. Materials and methods

2.1. Spore production

A 1:1 mixture of *P. infestans* mating type A1 and A2 was used in this study. The isolates were cultivated on ecological Pea Rye agar plates. Sporangia were harvested from agar plates with PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and stored at −20 °C until further use. *Botrytis cinerea*, *Epicoccum* sp., *Blumeria graminis*, *Tilletia tritici*, *Melampsora euphorbia* representing *Ascomycetes*, *Deuteromycetes* and *Basidiomycetes* with air-borne spores were used for cross-reactivity studies.

2.2. Phytophthora mAb

A mouse monoclonal antibody (phyt/G1470) raised against *P. infestans* mycelium was a kind gift from Agdia Incorporated (Elkhart, Indiana, USA). The mAb was received as an ammonium sulphate precipitate and was further purified by proteinA affinity chromatography.

2.3. Immunofluorescence microscopy

P. infestans sporangia were washed from agar plates and 20 μl of the spore suspension (5×10^5 spores in 1 ml water) were applied to multiwell glass slides and allowed to germinate for 16 h at room temperature. The glass slides were dried in a laminar air flow hood, followed by fixing with 20 μl of 3% paraformaldehyde for 30 min. Next the glass slides were incubated with 20 μl 1% (w/v) skimmed milk powder (Sigma-Aldrich, St Louis, USA) in PBS for 30 min. Three-fold dilution series of phyt/G1470 mAb in PBS-T (PBS containing 0.1% (v/v) Tween20) were made in the range 0.03–8 μg/ml. Twenty microliters of each phyt/G1470 mAb solution was applied to each well and incubated for 60 min. A 2.7 μg/ml phyt/G1470 mAb solution was found to give the best fluorescence intensity with limited background fluorescence. Next 20 μl FITC (fluorescein-5-isothiocyanate)-conjugated goat anti-mouse IgG immunoglobulin (Sigma-Aldrich) diluted 1:64 in PBS-T was added and incubated for 60 min. Following each step the glass slides were washed 3 times for 5 min in PBS-T. The glass slides were dried and one drop of CITIFLUOR (AGAR Scientific Limited, Essex, England) was added to each well and a coverslip was applied covering all wells. The fluorescence was observed with an UV Leitz Laborlux S microscope and documented using a camera connected to the microscope. Control wells were included, in which phyt/G1470 mAb was replaced with PBS-T, but otherwise treated similarly.

2.4. Cross-reactivity studies by plate-trapped antigen ELISA (PTA-ELISA)

Each spore species suspension (100 µl of 5×10^5 spores/ml) in PBS was added to each well in MaxiSorp™ plates (Nunc Glostrup, Denmark) and incubated for 16 h at 37 °C, followed by blocking with 200 µl 1% (w/v) skimmed milk powder in PBS. Wells were washed with PBS-T and 100 µl phyt/G1470 mAb (1 µg/ml in PBS-T) was added. Alkaline phosphatase (AP)-conjugated polyclonal goat anti-mouse IgG+IgM (Immunokontakt, Wiesbaden, Germany) diluted 1/2500 in PBS-T was added to PBS-T-washed wells. The ELISA was developed using 100 µl/well of pNPP AP-substrate (Sigma-Aldrich) and incubated for 30 min in the dark at 37 °C. Each spore species was analysed in triplicate. Unless otherwise stated all steps were performed for 1 h at 37 °C.

2.5. Subtractive inhibition ELISA

Optimal assay conditions were identified by checkerboard ELISA. Microtitre plates were coated with 0.2 µg/well polyclonal rabbit anti-mouse IgG1 (SouthernBiotech, Birmingham, UK) in 100 µl PBS for 1 h at 37 °C. The coated wells were washed with PBS and blocked with 200 µl 1% (w/v) skimmed milk powder in PBS. Sporangia standards (200 µl of each concentration) were prepared by a three-fold dilution series in HBS (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.2% (v/v) Tween, pH 7.4) and phyt/G1470 mAb (200 µl of 0.1 µg/ml) was added yielding a final 0.05 µg/ml phyt/G1470 mAb concentration. The mixture was incubated for 60 min at 37 °C and inverted frequently to allow good mixing of sporangia and phyt/G1470 mAb. Sporangia-bound phyt/G1470 mAb was removed from the remaining free phyt/G1470 mAb by centrifugation at $1500 \times g$ for 5 min. The phyt/G1470 mAb-containing supernatants were carefully removed and added to ELISA plates in quadruple wells (100 µl/well) for each spore concentration. AP-conjugated goat anti-mouse IgG+IgM (Immunokontakt) diluted 1/2500 in PBS-T was added to the wells. Plates were washed with PBS-T and 100 µl of pNPP AP-substrate was added followed by incubation for 30 min in the dark at 37 °C. The absorbance was read at 405 nm. Unless otherwise stated all incubation steps were performed for 1 h at 37 °C. The average response from the quadruple measurements (A) was divided by the average response of phyt/G1470 mAb in HBS only (Ao) to give normalised values. Cross-reactivity studies were performed with serial dilutions of inhibiting antigen (*B. cinerea*, *Epicoccum* sp., *B. graminis*, *T. tritici*, *M. euphorbia*) alongside the same dilutions with *P. infestans* sporangia. Percent cross-reactivity (CR) was estimated using the expression: $CR = (C/C^*) \times 100\%$, where C and C^* are the IC_{10}-values for *P. infestans* and the reference spore, respectively.

2.6. Development of a Biacore®-based subtraction inhibition assay

2.6.1. Antibody immobilisation

Analyses were performed with a Biacore® 3000 sensor (Biacore®, Uppsala, Sweden) using filtered and degassed HBS running buffer and a CM5 dextran surface (Biacore®). The optimal pH for immobilisation of commercial goat anti-mouse IgG1 polyclonal antibody (SouthernBiotech, Birmingham, UK)

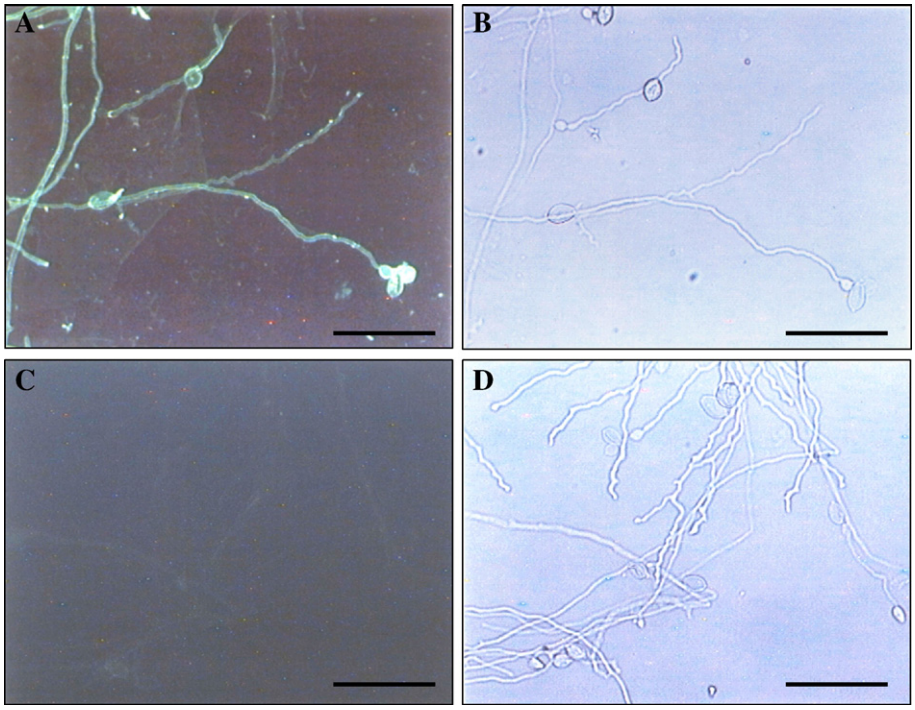

Fig. 1. Immunofluorescence microscopy using phyt/G1470 mAb. Germinated *P. infestans* sporangia were used as the antigen. Panel A shows the binding of phyt/G1470 mAb to sporangia and germ tubes under UV light. Panel B: the same as A, but viewed in normal light. Panel C: control well with phyt/G1470 omitted. Panel D: same as C but viewed in normal light. Fluorescence is observed from the sporangia and the protruding germ tubes. The control experiment is lacking fluorescence, thereby confirming the binding of phyt/G1470 mAb to sporangia. The black scale bar represents 100 µm.

was determined by diluting the antibody (10 μg/ml) in 10 mM sodium acetate buffers with pH from 4 to 5.5. Each dilution was injected (10 μl, flow rate 10 μl/min) over a blank flow cell in a CM5 chip and pH 4.5 was found to be optimal for immobilisation. The chip was activated by a 70 μl injection of a mixture of 50 μl of 100 mM *N*-hydroxysuccinimide (NHS) with 50 μl of 400 mM *N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) at a flow rate of 10 μl/min. Goat anti-mouse IgG1 was immobilised by injection of 100 μg/ml diluted in 10 mM sodium acetate, pH 4.5 at a flow rate of 10 μl/min over the activated dextran surface for 10 min. Unreacted sites were subsequently deactivated by injecting 1 M ethanolamine, pH 8.5 (Biacore®) at 10 μl/min for 7 min.

2.6.2. Surface regeneration

The surface was effectively regenerated using 30 μl glycine-HCl, pH 2.0 at a flow rate of 10 μl/min.

Surface regeneration and stability was investigated by repeated injections of 10 μl of 2.5 μg/ml phyt/G1470 mAb in HBS buffer at a flow rate of 5 μl/min followed by regeneration as above.

2.6.3. Assay setup

Sporangia concentration standards were made by serial dilutions in PBS (75 μl samples). Seventy-five microliters of phyt/G1470 mAb (5 μg/ml) in HBS buffer was added to each solution, giving a final mAb dilution of 2.5 μg/ml. The mixtures were incubated and centrifuged as described for the subtractive inhibition ELISA. One hundred microliters of each supernatant was carefully removed without touching the spore pellet. Ten microliters of each supernatant was assayed randomly in triplicate by automated analysis using the Biacore® 3000 instrument. The samples were injected over the CM5 chip surface at a flow rate of 5 μl/min and the resulting response unit change was measured at the end of each injection. Following each sample injection the surface was regenerated as described above. Mean values from triplicate measurements (*R*) and a blank sample response containing only phyt/G1470 mAb (Ro) were calculated and normalised values (*R*/Ro) were obtained, thereby obtaining the degree of inhibition for each spore concentration. The assay was repeated on three separate days and three times on the same day to generate interday and intraday variation data respectively. Calibration curves were constructed by fitting the data to a four-parameter logistic function using the Bia-evaluation software (Biacore, version 4.0.1). The detection limit was calculated as the lowest spore concentration resulting in 10% inhibition (IC_{10}) as suggested previously (Hennion and Barcelo, 1998).

3. Results

3.1. Sporangia binding of phyt/G1470 mAb

The mouse phyt/G1470 mAb used in this study was raised against *Phytophthora* mycelium. To verify that the phyt/G1470 epitope for the mAb was present on sporangia as well as mycelium structures, immunofluorescence microscopy was performed using germinated sporangia on glass surfaces. Fluorescence was observed from the sporangia and in the protruding germ tubes (Fig. 1, panel A). This fluorescence was not seen in the control experiment (panel C) and illustrates the suitability of phyt/G1470 mAb for *Phytophthora* sporangia detection.

3.2. Cross-reactivity profile of phyt/G1470 mAb

The phyt/G1470 mAb is the detection antibody in a commercial double antibody sandwich ELISA (DAS-ELISA), which detects *Phytophthora* to the genus level only. No cross-reactivity has been observed in the DAS-ELISA against isolates of *Aspergillus* sp., *Fusarium* sp., *Monilinia* sp., *Penicillium* sp., *Rhizoctonia* sp., *Sclerotinia* sp. and some *Pythium* sp. However, weak to moderate cross-reactivity has been observed against 10 *Pythium* species (www.agdia.com). To further expand the phyt/G1470 mAb cross-reactivity profile, additional isolates representing genera with air-borne spores were tested by PTA-ELISA. The reactivity towards *B. cinerea*, *B. graminis*,

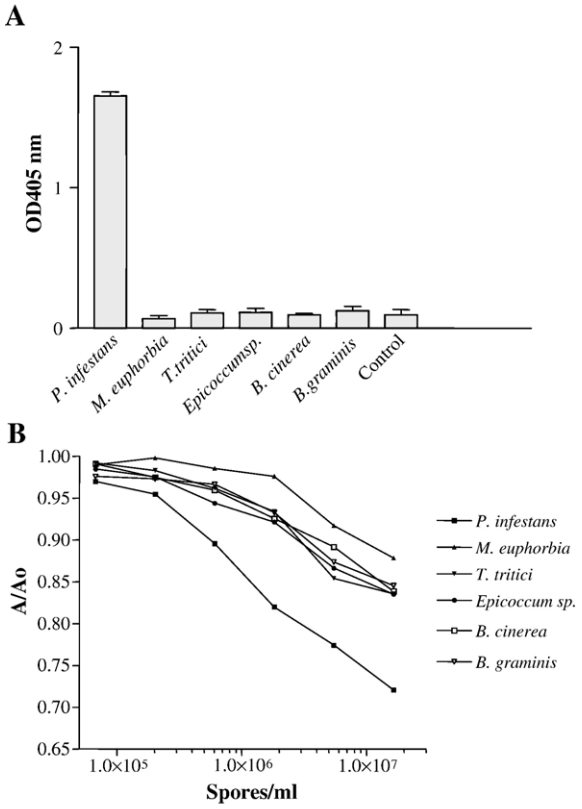

Fig. 2. Cross-reactivity of phyt/G1470 mAb. Panel A: The antibody was tested against five genera representatives (*B. cinerea*, *B. graminis*, *Epicoccum* sp., *M. euphorbia* and *T. tritici*) by PTA-ELISA. Reactivity of phyt/G1470 mAb was only found in *P. infestans* sporangia coated wells. Results shown are mean and standard deviations from triplicate measurements. Panel B: Cross-reactivity of phyt/G1470 mAb in a subtractive inhibition assay. Different spore species (*B. cinerea*, *B. graminis*, *Epicoccum* sp., *M. euphorbia* and *T. tritici*) were analysed alongside *P. infestans* sporangia. Normalised values (*A*/Ao) were plotted against the spore concentration. IC_{10}-values were obtained and the percentage-cross-reactivity estimated for each spore species. The following percent cross-reactivities were estimated: *M. euphorbia* (3.7%), *T. tritici* (11.1%), *Epicoccum* sp. (11.1%), *B. cinerea* (11.1%) and *B. graminis* (11.1%).

Epicoccum sp., *M. euphorbia and T. tritici* were found to correspond to that of the control background (PBS coat only), illustrating a high specificity of phyt/G1470 (Fig. 2A).

3.3. Evaluation of a subtractive inhibition ELISA assay

A subtractive inhibition ELISA (incorporating phyt/G1470) was set up for *P. infestans* detection. Fungal spores from *B. cinerea*, *B. graminis*, *Epicoccum* sp., *M. euphorbia* and *T. tritici* were analysed alongside *P. infestans* to investigate the cross-reactivity profile of phyt/G1470 in the subtractive inhibition assay (Fig. 2B). From the lowest spore concentration resulting in > 10% inhibition, IC_{10}-values could be obtained and the percentage-cross-reactivity estimated for each spore species. Weak cross-reactivity (~ 11%) was found with four species (*T. tritici*, *Epicoccum* sp., *B. cinerea* and *B. graminis*) but based on the PTA-ELISA results it is concluded that the reactivity is due to unspecific reaction of phyt/G1470 mAb with increasing spore concentrations. This supports the subtractive inhibition assay in terms of specificity.

3.4. SPR assay preparation

The subtractive inhibition assay was further implemented into a Biacore® 3000 SPR sensor. A goat anti-mouse IgG1 polyclonal antibody was immobilised to a CM5 chip surface (approximately 17,000 RU, data not shown). A 2.5 µg/ml solution of phyt/G1470 mAb gave an approximate binding response of 250 RU. The binding response was compared to that of a blank chip surface, which gave insignificant binding, thereby illustrating the specificity of the binding response (data not shown). Optimal regeneration conditions were investigated and a 30 µl pulse of 10 mM glycine-HCl, pH 2.0 at a flow rate of

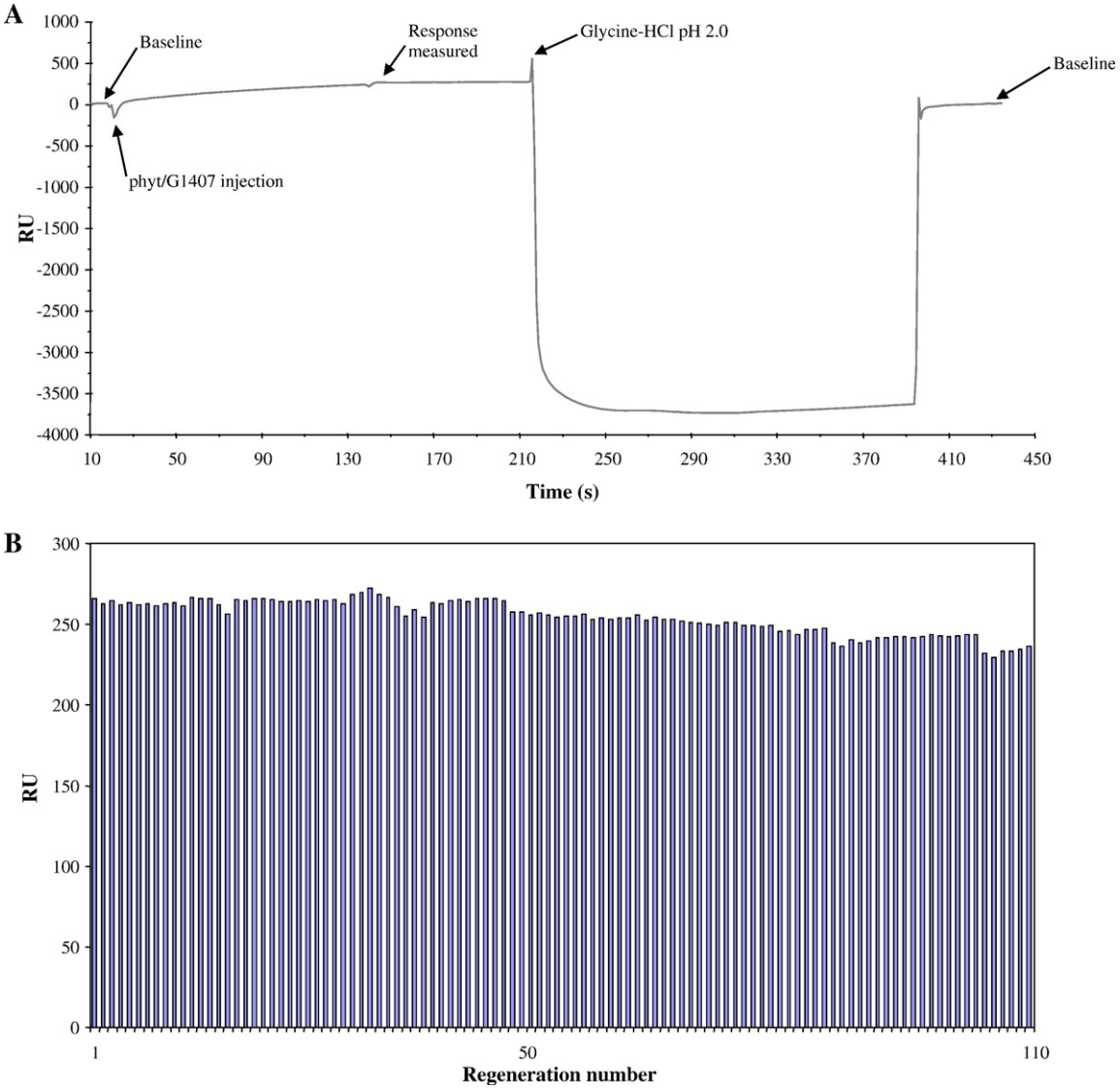

Fig. 3. Regeneration and surface stability. Panel A shows a complete binding and regeneration cycle using a 2.5 µg/ml proteinA purified phyt/G1470 mAb in HBS and glycine-HCl pH 2.0. After phyt/G1470 mAb injection the resulting response is measured and following regeneration the baseline response is restored. Panel B shows the surface stability in 110 binding and regeneration cycles. A decrease in surface activity of 6% from the first binding cycle (251.5 RU) to the last (236.4 RU) was observed.

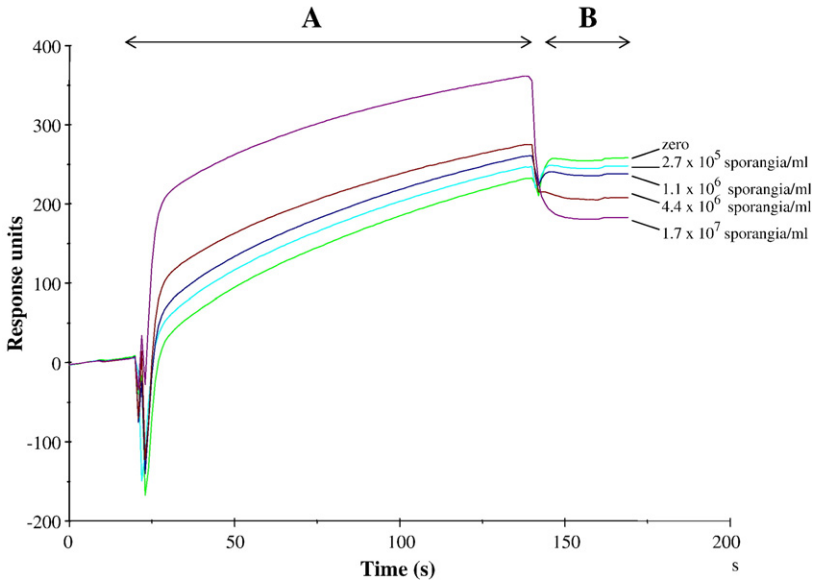

Fig. 4. Overlay plot from one assay demonstrating that the binding response is inversely proportional to the sporangia concentration. Representative results from sporangia concentrations ranging from 2.7×10^5 to 1.7×10^7 sporangia/ml are shown. Section A of the sensorgram is the injection phase and section B is the washing phase where the final response units are measured.

10 µl/min was shown to be sufficient for regeneration (Fig. 3A). To investigate long-term surface stability, repeated antibody binding and regeneration cycles were performed (Fig. 3B). This demonstrated that the surface could be regenerated more than 100 times with only a 6% decrease in the surface activity from the first to the last binding cycle. The chip surface was used throughout the analyses described in this paper and the surface activity did not decrease more than 20%.

3.5. SPR assay setup

The subtractive inhibition assay was performed by incubating decreasing concentrations of sporangia with phyt/G1470 mAb. The spore-bound phyt/G1470 mAb was removed by centrifugation and the remaining unbound mAb was subsequently quantified using the goat anti-mouse IgG1 CM5 surface and the Biacore® 3000 instrument. As seen from the representative overlay plot in Fig. 4, a binding response inversely proportional to the spore concentration was obtained, thereby verifying the biosensing principle. Sporangia standards were made and analysed randomly in triplicate alongside a zero sample containing phyt/G1470 mAb only. The normalised values were fitted to a four-parameter logistic function to construct a calibration curve showing correlation between degree of inhibition and sporangia concentration (Fig. 5). The range of detection was found to be approximately 1.4×10^5–3.6×10^7 sporangia/ml and based on the IC_{10}-value a detection limit of 2.2×10^6 sporangia/ml was achieved. The total analysis time, including sporangia-mAb pre-incubation, centrifugation and Biacore® analyses, was approximately 75 min.

3.6. SPR assay performance

The assay stability was investigated by interday and intraday variability studies. The coefficients of variation (CV's) for each point of the standard curves were found to be in the range from 0.09 to 5.47% and 0.48 to 4.83% for the interday and intraday studies respectively (Table 1). This illustrated good reproducibility of the immunoassay. The percentage accuracy between the fitted four-parameter logistic function and the actual concentration used was determined and the data fitted to the curves with percent accuracies ranging from 80.18 to 128.6% in the interday analysis and 72.07 to 142.15% for the intraday

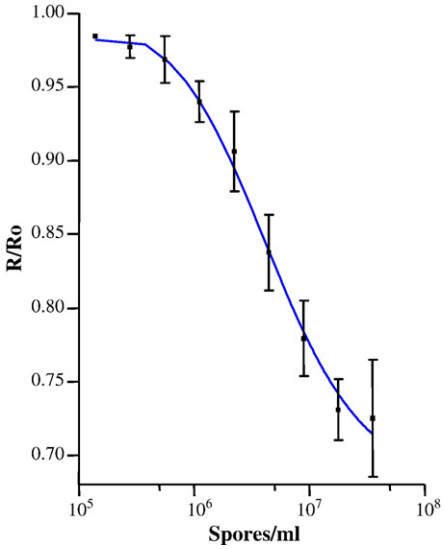

Fig. 5. Interday calibration curve. *P. infestans* sporangia standards in the range 1.3×10^5–3.5×10^7 were prepared and analysed in triplicate on 3 different days. The normalised responses (R/Ro) were plotted against the sporangia concentration to illustrate the degree of inhibition. Means and standard deviations are shown. The data was fitted to a four-parameter logistic function (blue line). From the IC_{10}-value a detection limit of 2.2×10^6 sporangia/ml was achieved. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Coefficients of variation (CV's) and percent accuracies from interday and intraday studies

Concentration (sporangia/ml)	Interday			Intraday		
	Back-calculated concentration (sporangia/ml)	CV's %	% Accuracies	Back-calculated concentration (sporangia/ml)	CV's %	% Accuracies
3.55E+07	2.76E+07	5.47	128.6	3.11E+07	2.81	87.60
1.78E+07	2.22E+07	2.84	80.18	1.72E+07	2.01	96.98
8.88E+06	8.62E+06	3.27	103.02	9.95E+06	1.60	112.05
4.44E+06	4.45E+06	3.03	99.78	4.40E+06	3.85	99.12
2.22E+06	2.11E+06	3.00	105.21	1.99E+06	4.83	89.76
1.11E+06	1.25E+06	1.48	88.8	1.19E+06	2.80	107.05
5.55E+05	5.51E+05	1.64	100.73	7.88E+05	1.96	142.15
2.77E+05	2.99E+05	0.79	92.64	2.00E+05	1.09	72.07
1.39E+05	*	0.09		*	0.48	

Interday % CV's (% CV = (standard deviation / mean) × 100%) were obtained from 3 assays performed on three different days, while the intraday CV's were from 3 assays performed on the same day. The percent accuracy was calculated using the expression ((actual sporangia concentration / back-calculated sporangia concentration) × 100%). (*) Refers to that no data could be obtained because the data point did not fit the calibration curve.

analysis (Table 1). This indicates that the four-parameter logistic function is an appropriate model to describe the immunoassay.

4. Discussion

Several mAbs have been found to target epitopes that are confined to specific fungal structures. This has resulted in the production of several mAbs that detects mycelium but not spores from the same species (Koistinen et al., 2000; Xia et al., 1992). phyt/G1470 mAb was raised against mycelium but by immunofluorescence microscopy it was found that phyt/G1470 mAb did target an epitope present in both mycelium and sporangia and was therefore found suitable for sporangia detection. phyt/G1470 mAb has been reported to be highly specific for species of the *Phytophthora* genera when used in the DAS-ELISA from Agdia (www.agdia.com). It is a common finding that mAbs are only specific to the genus level only (Werres and Steffens, 1994), however a low cross-reactivity has also been observed for phyt/G1470 mAb towards some *Pythium* species. This indicates a shared epitope for phyt/G1470 mAb within *Phytophthora* and *Pythium* species reflecting the close taxonomic relationship between the Oomycetes *Phytophthora* and *Pythium*.

To expand cross-reactivity studies of phyt/G1470 mAb, PTA-ELISA using spores from five additional representative fungal genera was performed. No cross-reactivity for phyt/G1470 mAb towards these species was found, further illustrating the high specificity of the mAb. phyt/G1470 mAb was incorporated in a subtractive inhibition ELISA for characterisation of the cross-reactivity profile of the assay setup. Limited cross-reactivity of the assay was observed and from IC_{10}-values percentage-cross-reactivity was found ranging from 4 to 11%. However, based on the PTA-ELISA results this cross-reactivity probably does not indicate a shared epitope for phyt/G1470 mAb in these species, but rather an unspecific binding of phyt/G1470 mAb to the increasing spore concentrations. Based on this knowledge, the specificity of phyt/G1470 mAb for *P. infestans* in the subtractive inhibition assay was underlined. However, as phyt/G1470 mAb displays affinity for other members of the *Phytophthora* genera, the subtractive inhibition assay is probably not exclusively specific for *P. infestans*, but further investigations will have to be performed in order to fully establish this.

Current *P. infestans* detection assays have analyses times from a minimum of 4 h (ELISA) up to one day for PCR methods as DNA-extraction is necessary (Bailey et al., 2002; Bohm et al., 1999; Gough et al., 1999; Harrison et al., 1990; Ristaino et al., 1998; Schlenzig et al., 1999; Tooley et al., 1997). The subtractive inhibition ELISA for *P. infestans* detection has an analysis time similar to that of existing ELISAs. The subtractive inhibition assay was implemented in a Biacore® 3000 sensor, which is fully automated, has precision liquid handling and integrated microfluidics. This allows for rapid and precise analyses of multiple samples, thereby decreasing analysis time (75 min in this study) and reducing the hands-on-time. In addition Biacore® assays have repeated analysis on the same functionalised surface, which reduces the assay price, if suitable regeneration conditions can be identified. The anti-mouse IgG1 antibody was immobilised on a CM5 chip and suitable regeneration conditions identified. During regeneration care should be taken not to affect the activity of the ligand, as the lifetime of the sensor surface can be compromised. This is done by identifying a regeneration solution that removes the analyte without harming the ligand. A single injection of 10 mM glycine-HCl, pH 2.0 was sufficient to remove phyt/G1470 mAb from the chip surface. The effect of low pH buffers is believed to be due to a reversible partial unfolding of the ligand and analyte making them positively charged, resulting in molecule repulsion as the binding sites no longer match (Andersson et al., 1999). Excellent long-term surface performance was found by repeated phyt/G1470 mAb binding and regeneration cycles, as 110 regenerations resulted in a 6% surface activity decrease. During the course of this study surface activity did not decrease more than 20%, which is the recommended cut-off value that ensures validity in Biacore® assays with repeated surface regenerations (Wong et al., 1997).

The association phases on the representative overlay plot (section A on Fig. 4) show binding responses for sporangia containing samples that are proportional to the spore concentration. However, following the shift to HBS running buffer, a

binding response that is inversely proportional to the sporangia concentration is seen (Fig. 4, section B). The SPR instrument measures refractive index changes both on and near the sensor surface. This means that the sporangia concentration-dependent response in the injection phase is due to soluble sporangia surface components (proteins, carbohydrates etc.) passing within close proximity to the sensor surface. However when the shift to HBS running buffer occurs (Fig. 4, section B) only the specific binding of phyt/G1470 mAb is observed, thereby confirming the high specificity of the sensor surface.

The immunosensor had a detection range from 1.4×10^5 to 3.6×10^7 sporangia/ml and based on the IC_{10}-value a detection limit of 2.2×10^6 sporangia/ml was achieved (the *R*/Ro-value at this concentration was 0.906). Looking at the linear part of the curve it is evident that the dynamic range of the assay is narrow. Furthermore, *R*/Ro-values only decrease to approximately 0.70, which is surprising as the *R*/Ro-values would be expected to decrease as the sporangia concentration increases. This effect was also observed in subtractive inhibition ELISAs suggesting that the phenomenon is phyt/G1470 mAb-dependent. A plausible explanation is that at high sporangia concentrations the sporangia stick together, thereby making epitopes inaccessible to phyt/G1470 mAb.

Current ELISAs for *P. infestans* detection are designed to detect the pathogen mycelium within plant tissue before any disease symptoms appear (Harrison et al., 1990; Schlenzig et al., 1999). Therefore, these studies have not reported the sporangia detection limit. Comparing our detection limit to that of available subtractive inhibition assays for bacteria detection (in the 10^5 cfu/ml range), we find that our immunosensor is less sensitive (Haines and Patel, 1995; Leonard et al., 2004). Improvements of the assay performance in terms of detection limit and dynamic range may be achieved using other available *P. infestans* antibodies (Gough et al., 1999; Harrison et al., 1990; Schlenzig et al., 1999). However, changing the antibody could compromise the specificity of the immunosensor.

5. Conclusions

The data presented in this study represents the first immunosensor-based approach for *P. infestans* detection and a detection limit of 2.2×10^6 sporangia/ml was achieved. The immunosensor is currently confined to a laboratory environment but future work is focused on the transfer of the assay onto portable sensors, where the centrifugation step will most likely be substituted by a micro-filter separation step of sporangia-bound phyt/G1470 mAb. The analysis time of the Biacore® assay is superior to available *P. infestans* detection assays, with a total analysis time of approximately 75 min. In addition, the use of the automatic Biacore® 3000 system increases sample throughput as many samples can be processed in overnight runs.

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