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Improved immunological detection of S*pongospora subterranea*

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Abstract

The genus *Spongospora* has two members which are important pathogens of vegetables, *S. subterranea* f.sp. *subterranea* (*Sss*) and *S. subterranea* f.sp. *nasturtii* (*Ssn*). The close taxonomic relationship of these *formae speciales* is based on similar cystosori morphology. The potato disease powdery scab, caused by *Sss*, is difficult to control. The key control measure is avoidance, aimed at planting clean seed in clean soil. For the development of routine tests for the presence of the pathogen on tubers and in soil, a monoclonal antibody (MAb) was developed using *Sss* cystosori as immunogen. It detected less than one *Sss* cystosorus and recognised *Sss* material from many parts of the world. No cross-reactions with other *Plasmodiophoromycetes* including *Plasmodiophora brassicae*, *Polymyxa betae*, *Polymyxa graminis* and different *Streptomyces* species causing common and netted scab of potatoes were observed. A novel tuber sample test method was developed using a kitchen peeling machine. This detected two tubers with one powdery scab lesion each in a sample including eighteen uninfected tubers. When soil samples spiked with cystosori were tested with the MAb, different *Sss* infestation levels could be discriminated. *Ssn* cystosori gave absorbance values in ELISA as high as *Sss* cystosori, whereas fresh crook roots of watercress containing *Ssn* zoosporangia and plasmodia or mud from an *Ssn* infected watercress bed gave low absorbance values or no reaction. The potential of these findings for the development of a disease control management are discussed.

Introduction

The genus *Spongospora*, together with eight other genera, belongs to the division *Plasmodiophoromycota*, included in the Kingdom *Protozoa* (Braselton, 1995). As there is uncertainty about their systematic classification, the informal term Plasmodiophorids is used. Several Plasmodiophorids are important pathogens and most of the 'fungal' virus vectors belong to this group. The two most important members of *Spongospora*, *S. subterranea* f.sp. *subterranea* (*Sss*) and *S. subterranea* f.sp. *nasturtii* (*Ssn*) are the only 'fungal' virus vectors that are also plant pathogens. Both are obligate parasites and spread via zoospores. Their resting spores (cystosori) have a characteristic spongiform-like structure which enables them to survive for a long time in the absence of host plants.

Once infested with *Sss*, soils can remain contaminated for up to 10 years. No effective control measures are available (Pitts, 2000). Many current potato cultivars are highly susceptible to powdery scab (Merz, 2000a). Breeding for resistance as a long term strategy has only been started recently (Falloon et al., 1999). Currently the most reliable control measure is prevention, which, in its most extreme, means planting clean seed into uncontaminated land. The traditional visual inspection of normally unwashed seed tubers runs the risk of misidentification (Merz, 2000b). Symptomless tuber contamination is also a risk. The contamination levels of field soils are difficult to determine.

Although bioassays involving the baiting of soil samples with tomato (Merz, 1989) or potato (Wale et al., 1993) seedlings have been used, they are labour intensive and slow. Sensitive, rapid and reliable detection and quantification of cystosori of *Sss* (on tubers and in field soils) and of *Ssn* spores (in watercress bed soil) would be a useful tool to investigate the relationship between soil contamination levels and disease levels observed in the field, and also a great aid to the development of disease management strategies.

Serological and molecular techniques have been used to develop detection methods for *Sss* and *Ssn*. Harrison et al. (1993) described the development of an enzyme-linked immunosorbent assay (ELISA) with a polyclonal antibody (PAb) for the detection of *Sss* cystosori on potato tubers and Walsh et al. (1996) demonstrated detection in field soils with a PAb. Monoclonal antibodies (MAbs) produced against *Sss* zoospores did not detect cystosori (Wallace et al., 1995). Bulman and Marshall (1998) and Bell et al. (1999) designed molecular primers which detect small amounts of *Sss*, but detection in soil and quantification still need improvement. Most recently, van de Graaf et al. (2003) used real-time PCR with newly designed primers to detect and quantify *Sss* in soil, water and plant tissue samples. Down and Clarkson (2002) developed a sensitive PCR-based detection system for *Ssn* but tested it mainly on zoospores.

This paper describes the sensitivity and specificity of a new monoclonal antibody developed against the cystosori of *Sss*, and its potential for detection of *Sss* and *Ssn*.

Material and methods

Development of monoclonal antibodies

Preparation of antigen and immunization

Cystosori of *Sss* were used as antigen and obtained from potato cv. Bintje grown in Switzerland. The cystosori preparation was made as described in Walsh et al. (1996). A mixture of intact cystosori, enzyme-treated cystosori and cystosori which had been homogenized with a pestle and mortar, suspended in PBS at 10 mg ml^{-1}, was used to immunize female BALB/c mice (RCC Ltd, Füllinsdorf, Switzerland) three times sub-cutaneously with 3–6 week intervals between injections. Injections consisted of 50–150 µl of this spore suspension mixed with an equal volume of complete Freund's adjuvant for the first and with incomplete adjuvant for the subsequent injections.

Cell fusion and selection

Three to 5 days after the last boost injection, 3×10^7 spleen cells were fused with 5×10^7 myeloma cells (De St. Groth and Scheidegger, 1980). Fourteen to 26 days after the fusions, cell culture supernatants were repeatedly screened for *Sss*-specific antibodies in a plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) or by triple antibody sandwich ELISA (TAS-ELISA) with goat anti-mouse IgG-alkaline phosphatase for detecting reagent. For the latter test format, a polyclonal antibody from earlier studies on *Sss* was used (Walsh et al., 1996). For these screenings, spore suspensions (similar to those used for immunization) and *Sss*-infected potato tuber peelings were used as positive controls and healthy potato tuber peelings and extraction buffer were used for negative controls. Selected hybridomas that tested positive for resting spore solutions and for infected potato tubers were subcloned by limiting dilution.

Development of ELISA reagents

Monoclonal antibodies were produced *in vitro*. The IgG fraction was purified from cell culture supernatant by precipitation with saturated ammonium sulphate and subsequent Protein A affinity chromatography (Amersham Biosciences, Duebendorf, Switzerland). Labelling of IgG with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) was done according to the recommendations of the supplier of the enzyme. The reagents were optimized for use in the double antibody sandwich procedure (DAS-ELISA) using certified microtitre plates (Nunc Immuno Plates MaxiSorp F96, Gibco Ltd., Uxbridge, UK) and operating with a working volume of 200 µl per well (Clark and Adams, 1977).

ELISA

To test the performance of the MAbs, a double-antibody sandwich (DAS) ELISA was used. This was performed either by using the complete kit

(Art. No. 111172, BIOREBA) containing all reagents, buffers, substrate, microtitre plates and applying a working volume of 200 µl for all steps, or by using a modified protocol which is described below: The IgG (Art. No. 111112, BIOREBA) was diluted 1 in 1000 in 0.05 mol l^{-1} sodium carbonate buffer (pH 9.6) and pipetted into each of 2 wells (200 µl per well) of microtitre plates (Nunc Immuno Plate MaxiSorp F96, Gibco Ltd., Uxbridge, UK) and incubated overnight (16 h) at 4 °C. Subsequently the plates were incubated for 2 h at 37 °C with sample homogenates (150 µl per well, ground for 1 min in extraction bags ('Universal') using a hand homogenizer (both BIOREBA AG, Reinach, Switzerland) for cystosori and plant material or with pestle and mortar for soil and peat in TRIS-buffered saline containing Tween 20 (0.05%) and PVP (MW 25 kD, 2% w/v) (extraction buffer; pH = 7.4)). This was followed by IgG conjugated to alkaline phosphatase (Art. No. 111122, BIOREBA) diluted 1 in 1000 in TRIS-buffered saline containing Tween 20 (0.05% v/v), PVP (2% w/v), BSA (0.2% w/v) and $MgCl_2$ hexahydrate (0.02% w/v) (pH = 7.4, 150 µl per well) for 3 h at 37 °C. Finally the plates were incubated with substrate (2-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets; one per 5 ml) in 10% diethanolamine adjusted to pH 9.8 with HCl) (150 µl per well) at room temperature. The optical absorbance at 405 nm (A_{405}) was measured after 1 h with an Anthos Labtec HT2 microplate reader (Tech Gen International, London, UK) blanked against unused wells or appropriate uncontaminated material.

Sensitivity

A sub-sample of quantified cystosori material from potato cv. Bintje was adjusted to a concentration of 100 cystosori per 200 µl in extraction buffer and further diluted to obtain the following concentrations: 42.5, 8.5, 1.7, 0.34, 0.068 and 0.013 cystosori per 200 µl. The same cystosori material and uninfested soil were used to prepare a dilution series containing 8000, 6000, 4000, 2000, 1000, 500 and 100 cystosori g^{-1} soil. A sample of 1 g of each dilution was ground in 2 ml extraction buffer. Additionally a similar dilution series was made with cystosori alone.

The prepared soil sample containing 500 cystosori g^{-1} was compared to a naturally infested field soil from a powdery scab trial site and a peat substrate used for minituber production, which turned out to be heavily infested. Other commercial peat substrates were included as controls.

Specificity

Equal amounts (5 mg) of cystosori material collected by sieving (120 µm mesh size) scrapings from contaminated tubers from Switzerland, Denmark, Sweden, Scotland, Japan, New Zealand, USA and Peru and peelings from infected potatoes from France, The Netherlands, Chile and Ecuador (0.1 g) were ground in 5 ml extraction buffer.

Cross-reaction of the MAb with the main *Streptomyces* species pathogenic to potato; *S. scabies* (Lambert and Loria, 1989a), *S. acidiscabies* (Lambert and Loria, 1989b), *S. europaeiscabiei*, *S. stelliscabiei* (Bouchek-Mechiche et al., 2000) and *S. turgidiscabies* (Miyajima et al., 1998) causal agents of common scab, and *S. reticuliscabiei* (Bouchek-Mechiche et al., 2000) causal agent of netted scab was tested. Pure strains were grown in 5 ml of tryptone-yeast extract broth (Pridham and Gottlieb, 1948) for 48 h at 25 °C in the dark with shaking. The cultures were harvested by centrifugation at 3000 g for 10 min. The pellets were resuspended in 2 ml of ELISA extraction buffer.

The specificity of the antibodies was also tested against extracts from common scab lesions from three potato cultivars (Santé, Cardinal and Désirée) and against extracts from netted scab lesions from tubers of cv. Bintje. Extracts were prepared by cutting 1 g of lesion tissue from diseased tubers and grinding them with pestle and mortar in 5 ml of ELISA extraction buffer for 1 min. Then 200 µl of each preparation were pipetted into duplicate wells of a microtitre plate (Nunc Immuno Plate MaxiSorp F 96, Gibco Ltd., Uxbridge, UK). Positive controls were freeze-dried extracts of *Sss* cystosori (supplied with the ELISA-reagent from BIOREBA AG, Switzerland), cystosori material from different potato cultivars (prepared as 20 mg of cystosori material ground in 2 ml extraction buffer) and extracts from powdery scab lesions (prepared as described above for common scab). Negative controls included extracts from healthy tubers (prepared from symptomless tubers as

described for lesion extracts) and extraction buffer only (all 200 µl per well).

Further cross-reaction was tested using samples of roots infected with the closely related Plasmodiophorid organisms, *Plasmodiophora brassicae*, *Polymyxa betae* and *Polymyxa graminis* (0.1 g ground in 5 ml extraction buffer).

The ability to detect cystosori from *Ssn* was also tested. Crooks from infected watercress roots, dried, stored and microscopically checked for the presence of cystosori (5 mg per 2 ml), as well as crooks from fresh plants and sieved soil from the same watercress bed (1 g per 5 ml), were ground in extraction buffer.

Potato tuber sample test procedure

To evaluate a practical method for screening a sample of tubers for the presence of *Sss*, a commercial kitchen peeling machine (Lips Maschinen AG, Arni, Switzerland) was used. Different ratios of healthy tubers:peeled tubers with one lesion left per tuber, in a total of 20 tubers were processed: 20:0, 19:1, 18:2, 16:4, 12:8, 4:16. Each batch was processed in the peeling machine for 12 s whilst flushing with about 500 ml of tap water. Two samples of 200 µl from each treatment were then taken from the washings for ELISA.

Statistical analysis

The A_{405} values of the cystosori dilution series were analysed after ANOVA using Tukey's procedure (HSD) with $P = 0.05$ and $n = 6$ using the SYSTAT 10 program (Systat Software Inc., Richmond, California, USA).

Results

Development of monoclonal antibodies

From two fusions, 768 wells of fusion products resulted in 712 wells with growing colonies; 35 of them were positive in the initial screening when cell culture supernatant was assayed for *Sss*-specific antibodies. Most of the initially positive clones were either not stable or not specific for *Sss*. One hybridoma clone secreting *Sss* antibodies of subclass 2b was finally obtained by multiple cloning under limited dilution and used for all subsequent experiments.

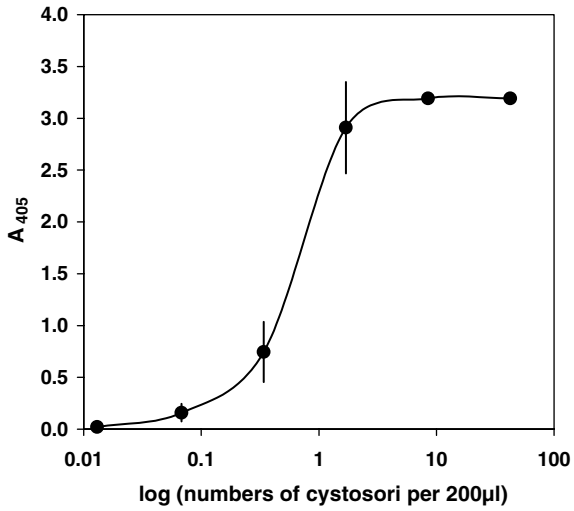

Figure 1. The relationship of the concentration of cystosori of *Sss* prepared from infected potatoes with A_{405} in DAS-ELISA. Mean data from 3 replicates and 2 ELISA plate wells each, blanked against negative control ($n = 6$). SD-bars.

Sensitivity

The quantified cystosori reacted strongly in DAS-ELISA. The data showed a linear relationship between 0.34 and 1.7 cystosori per well (Figure 1). The detection limit was at 0.068 cystosori per well. There was no significant difference between the A_{405} values for 0.013 and 0.068 cystosori ($P > 0.05$) , however the A_{405} for 0.34 cystosori was significantly higher than that for 0.068 ($P < 0.01$). When artificially infested (spiked) soil was tested, the sample with 100 cystosori g^{-1} was discriminated from the uninfested control, but with an A_{405} value ten times lower than the A_{405} value of the equivalent amount of cystosori without soil (Figure 2). Hence, the detection limit was less than 1 cystosorus ml^{-1} extract when testing purified cystosori, but approximately 100 cystosori g^{-1} soil when testing spiked soil. Samples from a heavily infested trial site ($A_{405} = 0.63$) and from peat substrate used for minituber production ($A_{405} = 0.50$) gave higher A_{405} values than the 500 cystosori g^{-1} soil sample ($A_{405} = 0.12$). Other peat substrates tested showed no reaction ($A_{405} < 0.005$).

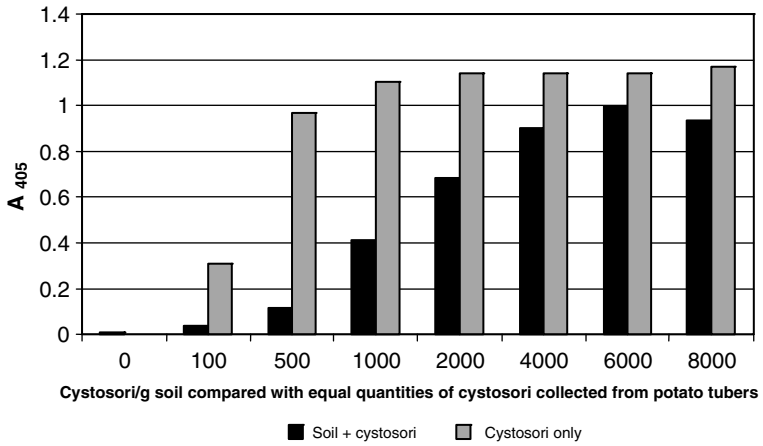

Figure 2. Absorbance (A_{405}) in DAS-ELISA of different numbers of cystosori of *Sss* in spiked soil samples compared with equal numbers of purified cystosori. Mean data from 2 wells.

Specificity

The *Sss* samples from Chile, Denmark, Ecuador, France, Japan, New Zealand, Peru, The Netherlands, Scotland, Sweden, Switzerland and USA gave similar positive A_{405} values. There was no reaction of the MAb with pure cultures of six *Streptomyces* species or common and netted scab lesion extracts, whereas the positive *Sss* controls gave high A_{405} values (Table 1). Likewise no reaction was obtained when *P. brassicae*, *P. graminis* and *P. betae* were tested.

Table 1. Absorbance (A_{405}) in DAS-ELISA of extracts from pure *Streptomyces* cultures or from peelings of tubers showing common and netted scab symptoms using the MAb produced against the cystosori of powdery scab (*Spongospora subterranea* f. sp. *subterranea*)

Sample	A_{405}[1]
Streptomyces species	
S. europaeiscabiei	−0.33[2]
S. stelliscabiei	−0.32
S. scabies	−0.33
S. acidiscabies	−0.34
S. turgidiscabies	−0.34
S. reticuliscabiei	−0.32
Fresh peelings from potatoes with:	
common scab	−0.33
netted scab	−0.32
Positive *Sss* controls:	
cystosori inoculum	2.31
fresh peelings from tubers with powdery scab	2.10
freeze-dried peelings from tubers with powdery scab	1.55
Negative controls:	
Fresh peelings from healthy tubers	−0.21
extraction buffer	−0.14

[1]Mean data from 2 wells.
[2]Negative values due to blanking against empty (unused) wells.

Dried and stored crooks from watercress roots containing cystosori of *Ssn* gave a similar A_{405} value compared to the same amount of a Swiss *Sss* cystosori homogenate (*Ssn* = 1.39, *Sss* = 1.43). Lower values were obtained when samples from fresh watercress roots with crook symptoms were tested (A_{405} = 0.31 and 0.06). Soil samples from the watercress beds where the infected plants had been taken, gave negative reactions.

Potato tuber sample test procedure

Only a slightly higher A_{405} value was obtained when 200 µl samples of flushing water originating from the tuber sample with the lowest ratio (19 healthy:one tuber with a single lesion) were tested, compared to the healthy control (HC = 0.022, 19:1 = 0.028). Two tubers with one lesion each together with 18 healthy tubers gave a clear difference (Figure 3).

Discussion

This paper describes a monoclonal antibody (MAb) produced against cystosori of *Sss* which

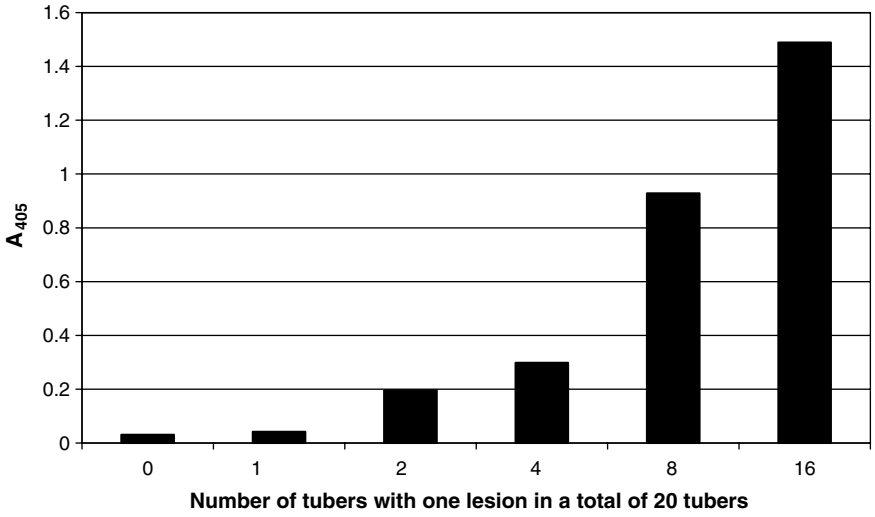

Figure 3. The A_{405} values in DAS-ELISA on batches of 20 potato tubers with different percentages of tubers peeled to leave one powdery scab lesion per tuber. The potato batches were processed in a potato peeling machine for 12 s and two samples (200 µl each) of the flushing water (500 ml) from each batch of potatoes were tested. Mean data from 2 wells.

detected *Sss* on tubers and in soil, but also verified the presence of cystosori of *Ssn*. Cystosori are the survival structures of both pathogens, found in soil and on tubers and transferred over long distances. Therefore, they are the main target for detection.

The sensitivity of the MAb in DAS-ELISA was similar to the highly sensitive polyclonal antibody (PAb), also produced against cystosori of *Sss,* tested in a PTA ELISA (Walsh et al., 1996). It detected less than one cystosorus which is sufficiently sensitive for the detection of tuber lesions. The advantage of a MAb is its unlimited supply and, related to this, its guaranteed reproducibility. Similar detection limits were found for PCR (Bell et al., 1999) and real-time PCR (van de Graaf et al., 2003).

The MAb provided a more linear relationship between the cystosori concentrations of less than 2000 g^{-1} soil in the spiked soils and A_{405} compared to that demonstrated earlier with the PAb (Walsh et al., 1996). In addition, the background reaction obtained from healthy soil was very low. But the comparison with the A_{405} values of the equivalent amounts of purified cystosori and cystosori in soil showed that in the presence of soil the detection limit of the MAb was slightly less than that of the PAb. This may have been due to the matrix effect of soil components interfering with the antigen–antibody interaction to some extent and thus masking the detection of cystosorus antigen. Similar inhibition problems with their real-time PCR assay were reported by van de Graaf et al. (2003) when spiked soil was tested. They detected inoculum levels <10 cystosori g^{-1} soil, but the soil seemed to be already naturally contaminated. With conventional PCR and spiked soil, Bell et al. (1999) could detect ≥ 5 cystosori g^{-1} soil consistently. Qu et al. (2000), using different primers and sieved soil (500 µm mesh), reported a detection limit of 4 cystosori g^{-1} soil. Preliminary experiments on the optimisation of the soil extraction method have shown that there is potential to further increase ELISA detection sensitivity and to improve significantly the current detection limit of 100 cystosori g^{-1} soil.

Both samples from the heavily infested soil and from one peat substrate gave much higher A_{405} values than the spiked soil sample with 500 cystosori g^{-1} soil. This supports the conclusion of Merz (1993) that heavily infested soils contain more than 500 cystosori g^{-1} soil. The heavy infestation of one of the peat substrates with *Sss* cystosori has been confirmed in a bioassay test that included other substrates and soils for comparison and all the appropriate controls (results not shown here). The findings are of special interest as this peat substrate is widely used for mini-tuber production. Here, the presence of any powdery scab infection means a big financial loss. This was one of the reasons why Rolot and Seutin (1999)

suggested a soil-less production system. Natural peat material, built-up over thousands of years, originates from places where potatoes have never been produced. The other commercial peat substrates tested turned out to be *Sss*-free. The possible cause of the contamination of this particular substrate therefore needs to be investigated in order to avoid spread of *Sss* via the peat substrate and mini-tubers grown in it. The results show that ELISA testing of peat substrates using the MAb described in this paper would provide a rapid and sensitive means of identifying *Sss* contaminated peat substrates prior to mini-tuber production.

Cystosori material from many countries and several continents were detected equally well by the MAb, demonstrating that they all possess the same epitope that is recognised by the MAb. Thus the MAb appears to have worldwide applicability.

A serious problem for the inspectors in the assessment of powdery scab infection levels on tubers is the similarity of symptoms caused by *Streptomyces* species which can be mistaken for powdery scab and *vice versa*, especially with unwashed material (Merz, 2000b). As the tolerance level for common scab is normally much higher than for powdery scab, there is a high probability that powdery scab infection levels of seed lots over the tolerance limit may go undetected and thus increase the risk of irreversible soil contamination. The availability of an antibody that has no cross-reaction with any *Streptomyces* species, recognised so far as pathogenic to potato and responsible for powdery scab like symptoms, makes it an efficient tool for routine detection and objective determination of powdery scab on tubers. In practice, representative sub-samples of potato tuber lots are normally tested. Instead of laborious hand peeling of every single potato tuber for ELISA, the potato peeling machine used can handle 20–30 tubers at a time and the operation is easy and takes only a short time. The potato tuber test method described is sensitive enough to detect a few lesions in a subsample of 20 tubers. Once standardised, such a test could be used to investigate how tuber infection levels relate to disease thresholds defined by certification schemes.

PCR assays also have the potential for detecting tuber infection. Bulman and Marshall (1998) stated that the most suitable method to rinse tuber scab samples prior to DNA extraction. Qu et al. (1998) obtained a PCR product with DNA from symptomatic tubers of different cultivars but not from micropropagated mini-tubers. Bell et al. (1999) extracted DNA from peel of scabbed and apparently healthy tubers but only material from classified seed or axenically micropropagated tubers gave no amplification product. Additionally, they tested washings of symptomless and moderately scabbed tubers and concluded that there were cystosori present in both extracts. None of them tested a routine detection method based on PCR.

Tests on the thick-walled resting spore stage of other members of the *Plasmodiophoromycetes* demonstrated that they do not share the protein epitope of *Sss* that our MAb reacts with.

The cystosori of *Sss* and *Ssn* share a similar morphology. They also showed similarity in their immunological reaction against the MAb. The epitope recognised could be a wall protein common in both types of cystosori. In contrast to this, both Bulman and Marshall (1998) and Bell et al. (1999) did not find much homology between the DNA sequences of parts of the ITS region of each organism and the *Ssn* specific primers designed by Down and Clarkson (2002) failed to amplify DNA from *Sss*.

The cystosori of *Sss* and *Ssn* are the main means of spread of powdery scab and crook root diseases respectively. Spread may result from the presence of the cystosori on potato tubers or by movement in contaminated soil. The cystosori are very resilient and able to survive in contaminated soil for many years in the absence of host plants. Consequently their detection and quantification are crucial for epidemiological studies and for control by disease avoidance (growing potatoes in fields where there are low levels, or no resting spores). *Ssn* cystosori can be found in roots of watercress plants as well as in the soil/mud in watercress beds; little is known about the role of the cystosori in the biology and epidemiology of crook root. Thus the new monoclonal antibody will provide a useful tool in future epidemiological studies on *Sss* and *Ssn* and for the first time will provide the opportunity for mass testing of potato tubers and field soils in order to control powdery scab. More information on soil infestation levels relative to powdery scab disease levels in the field is needed in order to define the necessary sensitivity range of a practical test method and also define threshold ELISA values for different levels of tuber infection.

ELISA tests are cost-effective and used routinely (mostly for virus detection) in potato seed certification schemes, this should facilitate rapid uptake of the ELISA test we have described with the new monoclonal antibody by potato seed testing organisations worldwide. Further development and optimisation of soil extraction methods and the potato tuber test procedure could improve the detection limits and efficiency of ELISA testing of soils and tubers. Additionally it may be possible to use the MAb in an immunocapture-based PCR test (Nolasco et al., 1993), thereby increasing the sensitivity of PCR detection.

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