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The use of molecular diagnostics to investigate the epidemiology of potato diseases

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Abstract. In recent years, quantitative molecular diagnostic assays based on real-time PCR have been developed for many pests and pathogens of potato. In addition, simple sequence repeat markers have been developed and used to track isolates of *Phytophthora infestans*. These diagnostic assays are now being used as tools to study unresolved questions in the epidemiology of potato diseases including late blight, powdery scab and black dot. Examples of various investigations designed to examine the relative contribution of seed and soil-borne inoculum in causing black dot and powdery scab on progeny tubers, the effect of environmental factors on the incidence and severity of powdery scab and the survival of asexual and sexual inoculum of *P. infestans* in soil are described. Consideration is given to the development of appropriate diagnostic assays, their use in conjunction with relevant and robust sampling techniques, and the interpretation of results to inform disease risk assessment and control strategies through industry collaboration.

Additional keywords: black dot, inoculum, PCR, powdery scab, real-time PCR, risk assessment, seed-borne, soilborne.

Introduction

Potatoes are subject to attack from a wide range of seed-borne and soilborne fungal diseases. In order to develop effective strategies for minimising disease risk in potato production, it is necessary to understand the epidemiological factors governing the incidence and severity of disease. Examples include the methods of transmission of the pathogen, the relative importance of different sources of inoculum, and factors influencing infection and the subsequent development of disease symptoms. Current knowledge of the epidemiology of various potato diseases varies according to both their historical and perceived importance and also to ongoing changes in the pathogen population such as increased levels of fungicide resistance, or selection for virulent strains. The gaps in our knowledge of the epidemiology of potato diseases can be filled partly by the development and application of novel molecular diagnostic tools. The use of molecular diagnostics to study potato disease epidemiology is discussed and detailed examples are given for three important potato diseases, late blight, powdery scab and black dot.

Development of molecular diagnostic tools

There are many unresolved questions in the epidemiology of potato diseases. Work has been hindered in some cases by the inability to detect low levels of the pathogen, including early or symptomless infections. In addition, methods are needed to accurately quantify pathogen levels since visual assessment can be subjective. In recent years, it has become possible to use specific diagnostic assays to address some of the outstanding questions. Diagnostic assays for a wide range of potato pathogens including *Phytophthora infestans* (Hussain *et al*. 2005), *Spongospora subterranea* (van de Graaf *et al.* 2003) and *Colletotrichum coccodes* (Cullen *et al*. 2002) have been developed at the Scottish Crop Research Institute (SCRI) over several years. In addition,

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co-dominant microsatellite (SSR) markers have been developed and used to track isolates of *P. infestans* for population biology and epidemiological purposes (Lees *et al.* in press).

The development of reliable, specific and quantitative assays for the detection of potato pathogens and their use in epidemiological studies is discussed. Specifically, the use of diagnostics to investigate the extent of pathogen contamination in potato stocks, the factors affecting disease development during growth and storage and the relationship between inoculum load and disease risk is considered. This paper is intended as an overview of the types of research made possible through the implementation of diagnostic assays. Specific examples of work carried out to understand aspects of the epidemiology of late blight, black dot and powdery scab, as outlined below, and current work to transfer diagnostics for direct use by the industry for risk assessment and decision making purposes, as addressed in the Discussion, are given.

Specific and quantitative diagnostic assays based on real-time (Taqman) PCR have been developed at SCRI for a wide range of pests and pathogens. A selection of assays for seed and soilborne pathogens of potatoes is listed in Table 1. In each case, specific primers were designed within the internal transcribed spacer (ITS) regions of the rDNA gene repeat. ITS regions are commonly used targets for diagnostic assays as they are less conserved than other regions and are present in multiple copies, which improves assay sensitivity (White *et al*. 1990; Bonants *et al*. 1997). Moreover, the availability of ITS sequence databases facilitates the alignment of sequences of a wide range of species for the selection of species-specific primers. Assays based on ITS sequence differences were designed to identify individual species. In the case of *Rhizoctonia solani*, primers were specifically designed for the identification of anastomosis group 3, the AG group representing 95% of isolates found on potato in the UK (Woodhall 2004). Real-time PCR is a modified PCR technique that uses two primers and an additional dual-labelled fluorogenic probe to allow the continuous monitoring of amplicon synthesis during thermocycling, and requires no post-PCR sample handling for target quantification (Orlando *et al*. 1998). Assays were developed to operate under standard conditions to allow multiplexing and have been tested for their sensitivity and specificity in detecting the target pathogens from plant and soil samples.

To ensure optimal quantification of pathogens from plant and soil samples, sampling and processing methods must be considered. In order to transfer the diagnostics to industry for disease risk assessment purposes, close attention must also be paid to developing a robust sampling strategy that it suitable for practical use. Following a review of the current literature and advice from Biomathematics and Statistics Scotland, protocols for sampling tubers in store and soil in the field have been written and are being tested. A full description of the methods used for extraction of DNA from plant samples can be found in the papers cited in Table 1. The soil extraction method currently being employed allows DNA to be extracted from a 60 g sample of field soil taken from an initial sample of 1 kg which consists of 100 × 10 g cores taken in a W pattern across a 4 ha field.

In addition to diagnostic tests based on real-time PCR, microsatellite markers for characterising *P. infestans* isolates have also been developed (Lees *et al.* in press). Microsatellites or simple sequence repeats (SSRs) are tandemly repeated motifs of 1–6 bases found in the nuclear genomes of all eukaryotes tested and are often abundant and evenly dispersed (Tautz and Renz 1984; Lagercrantz *et al*. 1993). Microsatellite sequences are usually characterised by a high degree of length polymorphism, and are ideal single-locus, objective co-dominant markers for genetic studies. The strategic and sustainable deployment of resistance requires knowledge of the extent of pathogenic variation in existing *P. infestans* populations. The main aim of our work in this area was to design and validate a set of polymorphic SSR markers for *P. infestans* genetic analysis and to employ combinations of those markers to assess their potential for assessing genetic variation on

Table 1. Conventional and real-time PCR assays developed at the Scottish Crop Research Institute for the detection of seedborne and soilborne potato pathogens

Disease	Pathogen	PCR assay	Real-time assay	Reference
Dry rot	*Fusarium* spp.	✓	✓	Cullen *et al.* (2005)
Black dot	*Colletotrichum coccodes*	✓	✓	Cullen *et al*. (2002)
Powdery scab	*Spongospora subterranea*	✓	✓	van de Graaf *et al*. (2003)
Black scurf	*Rhizoctonia solani* AG3	✓	✓	Lees *et al.* (2002)
Silver scurf	*Helminthosporium solani*	✓	✓	Cullen *et al.* (2001)
Common scab	*Streptomyces scabies*	✓	✓	Cullen *et al.* (2000)
Gangrene	*Phoma foveata*	✓	✓	Cullen *et al.* (2000)
Watery wound rot	*Pythium ultimum*	✓	✓	Cullen *et al.* (2000)
Pink rot	*Phytophthora erythroseptica*	✓	✓	Cullen *et al.* (2000)
Late blight	*Phytophthora infestans*	✓	–	Hussain *et al*. (2005)

a European and global scale as part of a collaborative project (www.eucablight.org). However, the reliable and rapid molecular markers also allow fingerprinting of isolates and their tracking in experimental work and as an aid to population monitoring to investigate short (field to field) and long range (country to country) spread of the pathogen. Experiments using SSR markers to investigate the effect of various factors on population composition are briefly outlined.

Late blight

Epidemiological knowledge gaps

Late blight caused by *P. infestans* is one of the most devastating diseases of potato worldwide (Stevenson *et al*. 2001) and, as such, has been extensively studied. However, the relatively recent migration of the A2 mating type of *P. infestans* from its presumed centre of origin, central Mexico (Niederhauser 1991; Goodwin *et al*. 1994), to other parts of the world during the 1970s and 1980s has resulted in increased disease severity and has refocused attention on the disease (Fry *et al*. 1993; Goodwin 1997). The occurrence of both mating types of *P. infestans* has resulted in the production of sexual oospores (Goodwin and Drenth 1997) that allow survival of the pathogen in the soil. The full role of the oospore phase of the pathogen's life cycle in the epidemiology of late blight has not yet been demonstrated and detection of oospores in soil is, therefore, desirable.

In addition, questions regarding the importance of tuber-borne infections, particularly symptomless infections, in initiating disease epidemics still remain. Infected seed tubers are an important means of transmission of *P. infestans* between potato crops (Boyd 1974) and the risk of disease and yield loss can be reduced by post-harvest or pre-planting visual disease assessments. Disease inspection reveals only established infections and the contribution of latent infections or tuber contamination to tuber loss in store and subsequent plantings is unknown. Therefore, it is important to be able to reliably identify and detect *P. infestans* in commercial tuber stocks and soil and to understand the role of oospores in the survival of inoculum under natural soil conditions.

Application of molecular diagnostics

Survival of inoculum. The ability of different types of *P. infestans* inoculum to survive over time was investigated (Hussain *et al*. 2005). Asexual (sporangia and mycelium) and sexual inoculum (oospores) was produced by inoculating detached potato leaflets (cv. Desirée) with either an A1 or A2 isolate or with a mixture of both isolates. After incubation for 2 weeks in a growth chamber (15°C, 12 h daylight), diseased leaves from each treatment (A1 alone, A2 alone, A1 and A2) and non-inoculated control leaves were placed individually in 10 cm bags (ten replicates) constructed from 20 µm nylon mesh that were then closed by sewing. The bags were buried at a depth of 12 cm in a 2 m^2 area of a field at SCRI in a randomised block design with four blocks. Eight leaves per treatment per block were buried to provide sufficient samples for eight quarterly samplings over a 2 year period starting in June 2000. The environmental conditions were monitored from the local SCRI meteorological site. At each sampling date, four replicate bags of each treatment, plus one non-inoculated leaf, were recovered and the leaf debris carefully removed. Deoxyribonucleic acid was extracted from 10 mg leaf material (Wang *et al*. 1993) and tested by conventional PCR. Sub-samples of the remaining debris from each bag were also examined under the microscope for the presence of intact *P. infestans* propagules, and the remaining debris was used in a viability assay.

Oospores were consistently detected using the PCR assay up to 24 months (total length of the study) after burial in soil, whereas the sporangial inoculum was detected for only 12 months after burial (Fig. 1). Sporangial inoculum was shown to be nonviable using a baiting assay, whereas leaf material containing oospores remained viable up to 24 months after burial. This assay is now being used in conjunction with SSR markers to investigate the role of oospores in field soils in initiating epidemics.

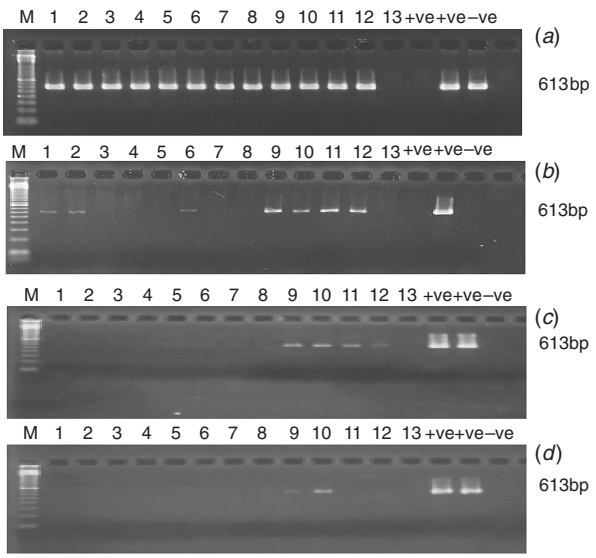

Fig. 1. Second round (nested) PCR amplification of *Phytophthora infestans* DNA from leaves inoculated with a single mating type or both mating types and recovered a) Six months b) 12 months c) 18 months and d) 24 months after burial in the field. Lanes 1–4, sporangia of A1 mating type; lanes 5–8, sporangia of A2 mating type; lanes 9–12, samples containing oospores after infection with both mating types, lane 13 uninoculated control. M represents 100 bp size marker. +ve/−ve represent positive and negative PCR controls.

Effect of control methods on P. infestans populations. Thirty isolates of *P. infestans* were characterised genotypically using SSR markers and also for mating type, fitness and metalaxyl resistance. A sub-set of five isolates was then selected. Field trials were carried out to assess the impact of host genotype and fungicide application on population structure, competitive ability of isolates and evidence for genetic exchange throughout the season. Five potato cultivars, having no known or minimal R-gene resistance to late blight, were planted in four plant plots with four replicates at two field sites in each of 2 years and were infected with the five individually characterised isolates. Each plant was sampled for blight lesions on four or five occasions during the late blight epidemic and isolates of *P. infestans* were isolated from individual leaf lesions and genotyped using SSR markers.

Results showed that the SSR markers were efficient in discriminating the five isolates used to inoculate the field trials and that the proportion of a given isolate retrieved from the trials was dependent on the host cultivar from which it was isolated. A full analysis of these results is underway. SSR markers have also been used to characterise parents in a cross between isolates in order to allow tracking to study the epidemiology of oospore infections in the field.

Powdery scab

Epidemiological knowledge gaps

Powdery scab, caused by *Spongospora subterranea*, has been poorly studied, in part due to the pathogen's intractability. Powdery scab can be a devastating disease in those areas of the world where environmental conditions are conducive, causing extensive losses in seed and ware crops (Wale 2000), while no totally effective control measures are available. *S. subterranea* forms persistent resting structures called cystosori or sporeballs, containing primary zoospores that can infect all underground structures of the host. Infected root systems may form zoosporangia which release more zoospores and, in potato and a few other hosts, develop galls containing cystosori (Würzer 1964; Jones and Harrison 1969; Andersen *et al*. 2002). Infected potato tubers can form cankers and scabs that attain a powdery appearance through the formation of masses of cystosori (Hims and Preece 1975). Many questions still remain in understanding the epidemiology of *S. subterranea*. For example, the role of non-solanaceous hosts in the incidence and severity of powdery scab is unclear (de Boer 2000). More recent work has helped to elucidate effects of temperature and moisture on disease incidence (van de Graaf *et al*. 2003), and clarified the relative importance of seed-borne or soilborne sources of inoculum (Wale 2000). Other areas of the biology of the pathogen, such as the effect of soil type on disease incidence and the occurrence of symptomless tuber infections are still poorly understood.

Application of molecular diagnostics

Inoculum source and effect of environmental factors on disease. Using the real-time assay in combination with conventional measurements of infection and disease symptom expression, the effects of soil inoculum level and three environmental factors (soil type, soil moisture regime and temperature) on the incidence and severity of powdery scab were investigated in potato plants grown under controlled environmental conditions. Full experimental details are given by van de Graaf *et al*. (2005). Symptoms of powdery scab on tubers were assessed visually, after which DNA was extracted from tuber peelings and quantified in a real-time polymerase chain reaction assay using primers and a TaqMan probe specific to *S. subterranea* (van de Graaf *et al*. 2003) to establish tuber infection levels.

In addition, the relative importance of seed-borne and soilborne inoculum was studied in a GB-wide trial where a stock of cv. Estima (SE1) with powdery scab was graded by hand to produce six levels of disease: 1. No visible symptoms, 2. 10% tubers diseased with $<1/8$ surface area diseased, 3. 20% tubers diseased with $<1/8$ surface area diseased, 4. 50% tubers diseased with $<1/8$ surface area diseased, 5. 20% tubers diseased, 4% with $>1/8$ surface area diseased 6. 50% tubers diseased, 10% with $>1/8$ surface area diseased.

The last two levels are above the Scottish certification standard. Sets of tubers were dispatched to 11 sites around Great Britain for planting in a randomised block design with two replicates within commercial ware crops. Each replicate plot comprised 50 tubers as two drills of 25 tubers. The field sites were chosen in the belief that they were free from soilborne inoculum of *S. subterranea*. All fields were irrigated. Tubers were harvested from plots within trials and assessed for incidence and severity of powdery scab.

Results of the controlled environment studies showed that soil inoculum concentration of *S. subterranea* did not significantly affect the incidence and severity of either tuber infection or powdery scab symptoms at maturity. No significant differences in disease incidence and severity were found between sandy, loamy and clay soils, although the two lighter soils yielded more powdery scab than clay soil. Constant dampness of the soil resulted in significantly more disease than a fluctuating moisture regime. Infection and disease levels were high at all three temperatures tested (9, 12 and 17°C), but symptoms were most severe at 12°C.

The use of the real-time PCR test has indicated that latent and immature tuber infections by *S. subterranea* could be common, especially under conditions sub-optimal for symptom development (Fig. 2). These infections

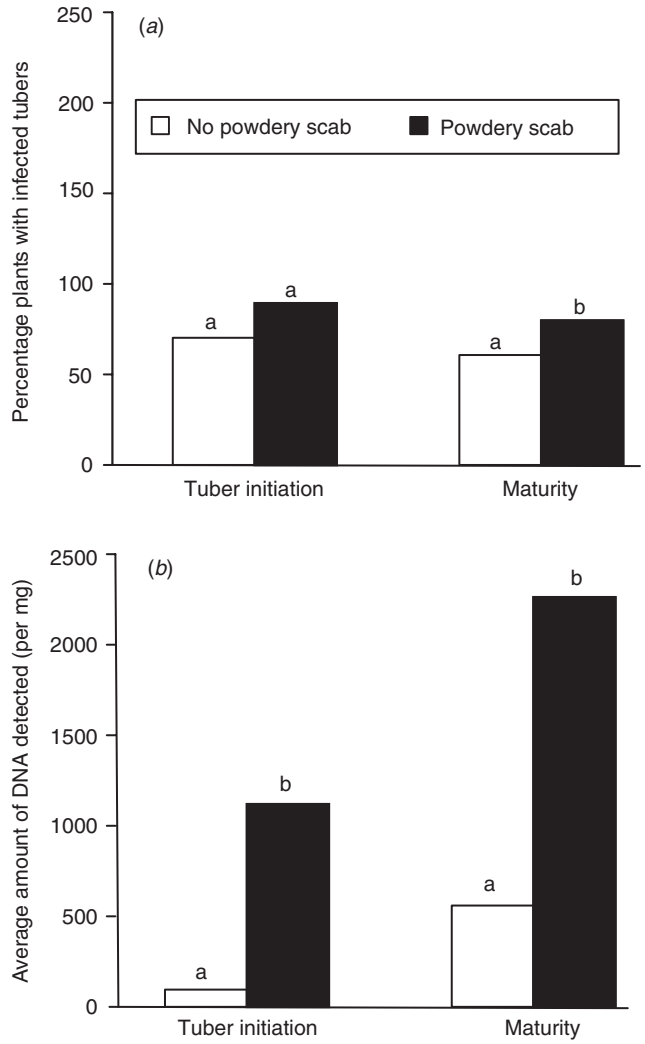

Fig. 2. Relationship between (*a*) visual symptoms of powdery scab on potato tubers (cv. Estima) at tuber initiation and maturity and (*b*) the incidence and amount of tuber infection (DNA/mg tissue) measured by real-time PCR. Differences between twinned columns with different letters are significant ($\alpha = 0.05$).

may continue to develop during storage and immature *S. subterranea* infections may even form sporeballs without acquiring the typical powdery appearance (Kole 1954). This could form an important source of inoculum, and there could, therefore, be implications for current seed inspection procedures and certification standards. The potential of latent infection appears to add to the risk of contamination of seed tubers as another potential source of the pathogen. For a full discussion of these results see van de Graaf *et al*. (2005).

Evidence from the field trials confirmed that there is no simple or consistent relationship between powdery scab inoculum on seed and disease developing on the progeny crop. Disease risk appears to be more related to the relative conduciveness of environmental conditions rather than inoculum level. Integration of control measures is likely to be the most effective way for growers to reduce the impact of the disease and this new information may be important for the prevention of powdery scab in potato growing areas around the world.

Black dot

Epidemiological knowledge gaps

In recent years, the growing market for fresh, pre-packed potatoes has resulted in an increase in the demand for washed potatoes with a high-quality appearance. As a consequence, skin-blemish diseases of potato including black dot (caused by *Colletotrichum coccodes*), silver scurf (caused by *Helminthosporium solani*) and black scurf (caused by *Rhizoctonia solani*), once considered to be of minor importance, are now viewed as serious problems. The British Potato Council estimates that black dot and silver scurf cause up to £5 million in losses to ware crops annually in the UK (Anonymous 1998), and there can be additional losses to the seed industry, particularly to the export market. It is also suspected that until recently, black dot symptoms on tubers were often mistaken for silver scurf symptoms, and that the importance and distribution of *C. coccodes* has, therefore, been underestimated. For more comprehensive reviews of black dot and silver scurf on potato see Lees and Hilton (2003) and Errampalli *et al*. (2001*b*), respectively. Many aspects of the life cycle of *C. coccodes* and the environmental conditions that favour black dot development are still unclear but the extensive distribution of the disease (Mordue 1967) suggests that infection can occur under a wide range of field conditions. Further work is needed to understand the extent of pathogenic variation in *C. coccodes* and to identify threshold levels of soil contamination as a precursor to field selection to avoid or reduce disease.

Application of molecular diagnostics

Role of seed and soilborne inoculum in causing disease on progeny tubers. To determine the relative contribution of different sources of inoculum in causing disease, experiments with contaminated seed and field soils were conducted.

Two field trials (Trial 1 and Trial 2) were carried out, each at two different sites. Prior to planting, soil samples were taken across the trial sites for quantification of soil contamination and a seed sample from each cultivar/disease category was taken for assessment of *C. coccodes* contamination. In the first trial, certified seed stocks of Maris Piper with black dot symptoms were sorted by hand into four different visual disease categories based on the surface area infection by black dot: 1 = no visual symptoms; 2 = <5%; 3 = 5–20% and 4 = >20%. Trials were established at two sites where soilborne inoculum was believed to be negligible, based on previous cropping history and were

subsequently shown, by real-time PCR, to be contaminated at an average level of 3 and 5 pg *C. coccodes* DNA/g soil, respectively, for site 1 and 2. Trials were laid out in a randomised block design with four replicates.

In the second experiment, trials were established at two sites with a history of black dot (subsequently shown to have average levels of 25 and 124 pg *C. coccodes* DNA/g soil (site 3 and 4, respectively)). In this case, certified seed stocks of Maris Piper without visual black dot symptoms were planted. Trials were laid out in a split plot design with irrigation as main plot and cultivar/azoxystrobin soil treatments as sub plots, with four replicates (only irrigation and azoxystrobin treatment means are presented here). At harvest, progeny tubers (45–85 mm fraction) were taken from each plot and assessed for *C. coccodes* contamination by visual assessment (50 tubers) and real-time PCR (24 tubers). The relationship between visual disease assessment of black dot on tubers and real-time PCR results was determined. *C. coccodes* DNA (pg/mL tuber sap) was quantified on contaminated progeny tubers. There was a strong positive relationship ($R^2 = 0.90$) between surface area infection (severity) based on visual assessment and the amount of *C. coccodes* DNA detectable by real-time PCR (Fig. 3). However, several samples displayed little or no visual sign of contamination, but had relatively high levels of detectable DNA (i.e. up to 130 000 pg DNA/mL tuber sap). This indicated that visual disease assessment could be used as a measure of disease severity in these experiments, without the need for testing using real-time PCR but that symptomless infections may be present.

Progeny tuber contamination was low at site 1 (3 pg/g soil), but higher at site 2 (5 pg/g soil) (see Fig. 4). At both sites the level of seed contamination had no significant effect (ANOVA; P = ns) on progeny tuber contamination. However, progeny tuber contamination was greatest in trial 2 when seed with no black dot symptoms was planted at the two sites with relatively higher levels of soil contamination (as indicated by the arrows in Fig. 4). Although these are preliminary

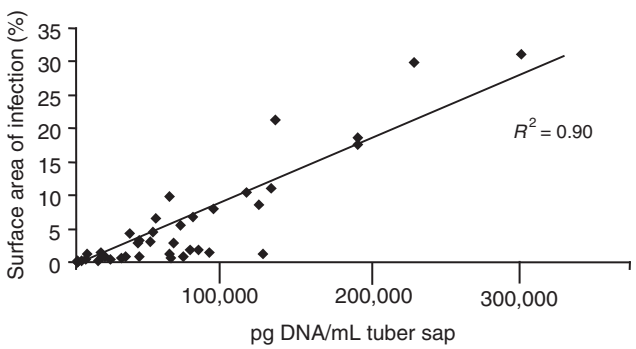

Fig. 3. The relationship between black dot severity (visual assessment) and contamination as determined by real-time PCR on progeny tubers. Each point represents a treatment mean in the four field trials.

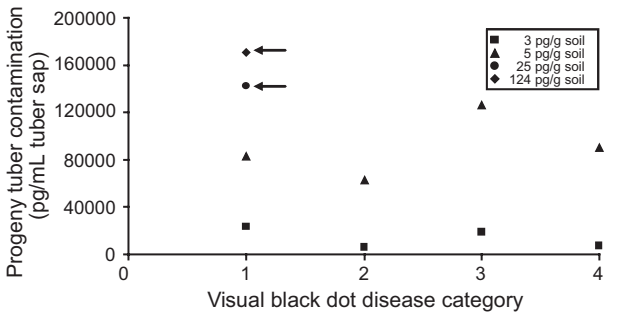

Fig. 4. *Colletotrichum coccodes* contamination of progeny tubers (pg DNA/mL tuber sap) resulting from various levels of seed (visual assessment) and soil (pg/g soil) contamination (as indicated by different symbols) in Maris Piper.

results, it appears that levels of field soil contamination play a greater role in causing disease on progeny tubers than seed contamination. Further work will be carried out to verify these results and to determine more accurately inoculum threshold for the development of black dot on progeny tubers.

Discussion

Soilborne diseases represent a considerable, but often unpredictable, risk for potato growers (Wale 2004). To control disease, it is necessary to have information to determine the potential for disease development: soilborne diseases present some additional challenges in the development of disease risk assessment and control strategies. Progress has been made in understanding the epidemiology of some soilborne fungal diseases as described in the examples given above. However, there has been insufficient transfer of these technologies to industry and disease management advice based on test results has not previously been available. When bringing together the knowledge from epidemiological studies with diagnostic testing, several issues must be addressed: data from diagnostic tests for soils and tubers must be related to gain maximum information for the grower and add to the scientific knowledge of disease epidemiology. As the distribution of pathogens in the field may be patchy, a robust sampling strategy must also be delivered. The relationship between soil inoculum levels and the incidence and severity of symptom expression, or symptomless infections, should be described, and the use of disease control methods linked to disease risk. Finally, soilborne pathogen diagnostics should be transferred to industry for validation on a wider scale.

Tests of our molecular diagnostic tools on a commercial scale over a range of cropping and environmental variables should confirm and increase the merit of their application. The wider use of these molecular tools will help to fill the remaining gaps in the knowledge of potato disease epidemiology and this, in turn, should improve disease risk assessment and contribute to a reduction in disease losses.

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