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Relative importance of seed-tuber and soilborne inoculum in causing black dot disease of potato

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Controlled-environment and field experiments were done to quantify the individual contribution of seed-tuber and soilborne inoculum of *Colletotrichum coccodes* in causing black dot disease of potato tubers. Seed-tuber and soilborne inocula of *C. coccodes* were quantified using an existing real-time PCR assay and related to subsequent incidence and severity of disease. In four field trials, a controlled-environment experiment and through the monitoring of 122 commercial crops, seed-tuber inoculum was found to be relatively less important than soilborne inoculum in causing black dot, and the level of seed-tuber inoculum did not significantly affect either the incidence or severity of disease or the percentage of progeny tubers deemed unmarketable. By contrast, soilborne inoculum had the potential to result in high levels of disease and the level of *C. coccodes* soil infestation (pg DNA g^{-1} soil) was found to have a significant effect. At soil infestation levels below 100 pg DNA *C. coccodes* g^{-1} soil, 7% of commercial crops had an incidence of black dot greater than 20%, increasing to 40% and 57% of crops at levels of 100–1000 pg g^{-1} and >1000 pg g^{-1} soil, respectively. These arbitrary threshold levels for soilborne inoculum related to disease risk are discussed. Interpretation of disease risk based on inoculum levels must, in the future, be informed by agronomic variables and potential control strategies.

Keywords: *Colletotrichum coccodes*, inoculum potential, real-time PCR, soil DNA extraction, *Solanum tuberosum*

Introduction

Black dot is a tuber blemish and foliar disease of potato caused by the fungal pathogen *Colletotrichum coccodes*, which can colonize all underground parts, basal stems (Andrivon *et al.*, 1997, 1998) and foliage (Mohan *et al.*, 1992; Johnson & Miliczky, 1993; Johnson, 1994) of the potato plant. Infection of tubers by *C. coccodes* results in the development of brown necrotic lesions on the tuber surface, characterized by the presence of black microsclerotia. Black dot has become one of the main blemish diseases affecting pre-packing potatoes in the UK; the reasons for this, and other aspects relating to both the pathogen and control of the disease were reviewed previously (Lees & Hilton, 2003).

Infection of progeny tubers by *C. coccodes* originates from inoculum on seed tubers (hereafter 'seed') (Jellis & Taylor, 1974) and from microsclerotia in the soil (Blakeman & Hornby, 1966). The pathogen is introduced into uncontaminated soil through seedborne inoculum and can survive in the soil as microsclerotia for at least 8 years (Dillard & Cobb, 1998). The extent of soil contamination, both in terms of geography and contamination level, is generally unknown, but believed to be widespread. In a survey of French potato-producing areas Andrivon *et al.* (1997) reported the presence of *C. coccodes* in all 37 soils tested using a baiting bioassay. Earlier work (Barkdoll & Davis, 1992) found that of 21 growers' fields sampled in Idaho in 1988, 18 (86%) were infested with *C. coccodes* at levels between 0 and 211 CFU g^{-1} air-dried soil.

In order to implement effective disease-management strategies for black dot it is important to understand the relative contribution of different inoculum sources in causing disease. Previous studies have investigated the roles of seed- and soilborne inocula, but the potential of naturally occurring levels of inoculum to cause disease under specific environmental and management conditions has not previously been ascertained because of difficulties in accurate quantification of seed- and soilborne inocula.

There is a general consensus that soilborne inoculum is of greater importance than seedborne inoculum in

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causing disease. Denner *et al.* (1998) demonstrated that soilborne inoculum doubled the incidence of disease on progeny tubers compared with seedborne inoculum. Read & Hide (1988) concluded that black dot can originate from both seed- and soilborne inocula, with the relative importance of each being dependent on disease severity or inoculum concentration.

More recently, Nitzan *et al.* (2008) showed that soilborne inoculum caused more disease than seedborne inoculum and described a non-linear relationship between soilborne inoculum (quantified as approximate numbers of sclerotia per litre of soil) and disease severity. In artificially inoculated trials, disease severity remained constant above an unquantified threshold of soilborne inoculum. No comparison of the response under natural field conditions was made.

There is limited evidence to show that crop rotation might be successful for controlling black dot. For example, Dillard & Cobb (1998) showed that 92% of sclerotia of *C. coccodes* buried 20 cm below the soil surface were viable after 8 years. Similarly, Cullen *et al.* (2002) were able to detect *C. coccodes* in naturally infested field sites from three UK sites that had not been planted with potatoes for 5, 8 and 13 years, and at trial sites used in the current work, DNA of *C. coccodes* was detectable in fields that had not been planted with potatoes since 1973. Hide & Read (1991) demonstrated that previous cropping did not affect the incidence of black dot. Therefore, of the disease-control measures available to growers, avoidance by planting healthy seed or avoiding contaminated fields is important. Visual identification of diseased seed stocks can be achieved, but detection of soilborne inoculum using a diagnostic test has not previously been available commercially.

The use of quantitative diagnostic tools in combination with appropriate sampling strategies and soil DNA extraction techniques means that the relationship between actual levels of seed and soil inoculum and disease can be investigated. Cullen *et al.* (2002) developed a real-time PCR assay and demonstrated that it could detect and quantify *C. coccodes* in DNA extracted from tubers and soil, but did not attempt to validate the assay on seed-tuber stock or in a field. Whilst reliable methods for extracting DNA from tubers exist, the accurate and consistent extraction of target DNA from soil has, in the past, proved problematic. Issues that can affect the accuracy of DNA-based soil testing include sampling strategies, sample size, DNA extraction from soil and assignation of disease risk categories and are discussed in detail by Ophel-Keller *et al.* (2008). Of these constraints, small soil sample size has been a major issue because the sample may not be representative of the field as a whole. By increasing the volume of soil processed from 10 to 60 g in a fine-milling protocol, direct extraction of soil DNA has been improved in such a way that when combined with the previously developed real-time PCR assays, accurate and robust quantification of soilborne *C. coccodes* is now possible (Brierley *et al.*, 2009).

This study was carried out to determine, quantitatively, the relative importance of the contribution of seed- and soilborne inocula to progeny tuber disease under controlled-environment and field-trial conditions and in commercial potato crops.

Materials and methods

Seed source, inoculum and experimental conditions – all experiments

Certified seed stocks (Super Elite, 35–50 mm) of the black-dot-susceptible potato cv. Maris Piper (resistance rating 4 on a 1–9 scale of increasing resistance) and the resistant cv. Saxon (resistance rating 7) (http://varieties.potato.org.uk/) were used in experiments 1 and 2. Minitubers, used in experiments 3 and 4, were sourced from GenTech Propagation Ltd. All seed was kept at 4°C in the dark until required. Tubers were washed and surface-sterilized by dipping in a 5% hypochlorite solution, rinsed thoroughly and air-dried before use. If seed tubers were not well sprouted prior to planting they were chitted (i.e. allowed to sprout) at 18°C under a 16-h-light/8-h-dark regime for approximately 14 days prior to use.

Inoculum was prepared for all artificially inoculated experiments using the following method. Three UK isolates of *C. coccodes* isolated from potato were grown on potato dextrose agar (PDA) in 9-cm^2 Petri dishes at 18°C in the dark for 16 days. An inoculum suspension containing all three isolates was then made by scraping the fungal colonies from 20 individual cultures into 1 L sterile distilled water (SDW). The concentration of the inoculum, which contained sclerotia, conidia and mycelium, was quantified as colony forming units (CFU mL^{-1}) by dilution plating.

Each controlled-environment experiment was conducted at 70% relative humidity with a 16-h-light/8-h-dark regime. All plants were grown in a peat-based SCRI compost in 3-L pots and were watered by hand into saucers to minimize cross contamination between pots and to maintain conditions of constant dampness as far as possible, unless otherwise stated.

Soil samples

For each crop a 4-ha portion of the field from which samples would be taken was identified. A soil sample was taken using a soil-sampling spear from the top 10 cm of soil of at least 100 points in a W-shape across the selected portion of the field to give a total of approximately 1 kg. The same approach was taken for field trials, but the area from which the samples were taken was restricted to that of the field trial.

Seed and progeny tuber sampling and assessment

For each seed-stock or seed-inoculum category used in controlled-environment experiments and field trials 24

seed tubers were assessed individually for *C. coccodes* contamination using real-time PCR as described below.

In field trials, progeny tuber assessments were made after harvest: 50 tubers per plot, 25 from the 45- to 65-mm fraction and 25 from the 65- to 85-mm fraction were placed in paper sacks and stored at 4°C for a month before being washed and assessed visually for black dot incidence and severity (percentage diseased surface area). The percentage of unmarketable tubers was calculated as the percentage of tubers with more than 10% surface area diseased.

For each monitored crop, both the seed-stock and progeny tubers were sampled as follows: a 50-tuber sample was assessed visually for disease and in addition, 100 tubers were subdivided into 10 replicate bulks of 10 tubers and each bulk sample assessed for *C. coccodes* contamination using real-time PCR as described below.

Real-time PCR: quantification of soil and tuber inocula

Where individual tuber testing was carried out the entire tuber was peeled and each sample processed separately. Where bulk tuber samples were used, a strip of peel was removed from one side of the tuber (from the rose end to the stolon end) with cores taken from both the stolon and rose ends. Samples taken from 10 individual tubers were then combined and each of the 10 bulk samples was assessed for *C. coccodes* contamination. In each case all tubers were washed and then peeled using a hand peeler. The peel from either the individual or bulk samples was sapped using a Pollahne Press and a 1·5-mL subsample of sap aliquoted into a 2-mL Eppendorf tube and kept on ice or frozen prior to further processing. The Pollahne Press was washed with 96% ethanol and 0·2 M NaOH between samples. A 0·5-mL aliquot of tuber sap was added to 1 mL extraction buffer in a 2-mL Eppendorf tube containing 0·2 g each of zirconia/silica beads and 1·0-mm glass beads, and was blended in a Mini-BeadBeater at 5000 r.p.m. for 60 s. Samples were kept on ice prior to DNA extraction and real-time PCR, which were carried out according to the methods of Cullen *et al.* (2001) and (2002), respectively. Each DNA sample was run in duplicate. The amount of *C. coccodes* DNA detected was expressed as ng DNA mL^{-1} tuber sap and values given are the mean of either 10 bulk samples, each containing peel from 10 individual tubers and having two replicates (i.e. mean of 20 values), or of 24 individual tuber samples in duplicate (i.e. mean of 48 values).

Soil DNA extractions were carried out according to the method of Brierley *et al.* (2009). In brief, individual 1-kg soil samples, collected as described previously, were thoroughly mixed. A 60-g subsample of soil was taken from each 1-kg sample and placed in a Retsch milling bowl (Planetary Ball Mill PM 400) with 120 mL extraction buffer and 12 ball bearings and milled at 300 r.p.m. for 5 min. Following milling, triplicate 1·5-mL aliquots were taken from each bowl and DNA extractions carried out on each. Bowls were cleaned with 96% ethanol and 0·2 M NaOH between samples to prevent cross-contamination. Duplicate samples from each DNA extraction were analysed using real-time PCR. Therefore, six readings were obtained for each soil sample and the mean value calculated (expressed as pg *C. coccodes* DNA g^{-1} soil).

Experiment 1: effect of seed inoculum level, temperature and moisture on black dot development under controlled environmental conditions

A certified seed stock of Maris Piper was sorted visually into four disease categories based on the percentage of tuber surface area covered with black dot symptoms: no visual symptoms; <5%, 5–20% and >20% surface area. Seed tubers from each disease category were grown in non-infested SCRI compost at either 18°C or 22°C under one of two watering regimes ('damp' = constant dampness maintained and 'dry' = half the water of the 'damp' treatment applied). All treatments had five replicates. Plants were grown as described previously for 70 days, after which time stems were cut off approximately 5 cm above soil level and watering ceased. The progeny tubers from each pot were harvested 18 days later and placed in a paper bag. The severity (percentage diseased area) of black dot on each individual tuber was recorded, and results expressed as the mean incidence and severity of black dot disease per pot. Individual tubers were washed and peeled, and peel from tubers within a pot was bulked. The quantity of *C. coccodes* DNA on progeny tubers was determined using real-time PCR.

Experiment 2: relationship between seedborne inoculum and black dot on tubers at harvest under field conditions

Two parallel trials were carried out in each of 2004 and 2005 using seed tubers from the same stock in each year. Trials were established on sites in England (Kings Lynn, Norfolk) and Scotland (Oldmeldrum, Aberdeenshire) where soilborne inoculum of *C. coccodes* was believed to be very low, based on cropping history. Site details are given in Table 1. Prior to planting, soil samples were taken across the trial sites and the amount of *C. coccodes* DNA quantified using real-time PCR.

Certified seed stocks of cv. Maris Piper showing black dot symptoms were sorted by hand into four different visual disease categories based on the percentage surface area covered with black dot symptoms: no visual symptoms; <5%, 5–20% and >20%. Trials were laid out in a randomized block design with four replicates. For all four field trials described here the plot size was four rows in ridges 11 m in length and tuber spacing was 25 cm. Harvested tubers were taken from the two inner rows. Outer guard rows were planted with seed from a disease-free stock of the same variety. All trials were managed as general ware crops with fertilizer, herbicide, late blight and aphid control and haulm destruction as per local practice.

Table 1 Details of potato field-trial sites (experiment 2)

	Location			
	Oldmeldrum Scotland	Kings Lynn England	Oldmeldrum Scotland	Kings Lynn England
Year	2004	2004	2005	2005
Soil type	Sandy clay loam	Silty clay loam	Sandy clay loam	Silty clay loam
Soil inoculum (pg DNA g^{-1} soil)	3	5	38	34
Previous potato crop	1999	1973	unknown	1973
Date of planting	25 May	25 May	3 June	5 May
Date of haulm destruction	14 September	8 September	1 September	6 September
Date of late harvest	26 October	20 October	10 November	19 October

Experiment 3: effect of soil inoculum level, temperature and cultivar on black dot development under controlled environmental conditions

Inoculum of *C. coccodes*, prepared as described previously, was added to batches of SCRI compost resulting in final concentrations of 0, 10, 99, 985 and 9552 CFU g^{-1} soil. Control treatments had an equivalent volume of water added. Minitubers of cvs Maris Piper and Saxon, shown to be free of *C. coccodes* contamination using PCR, were planted in the infested compost and grown at either 18°C or 22°C with four replicates in a randomized block design. Plants were grown for 72 days, after which stems were cut off and watering ceased. The progeny tubers were harvested 14 days later and were assessed as described in experiment 1.

Experiment 4: relationship between natural soil contamination level and black dot on progeny tubers

Thirty-two individual soil samples were collected from fields used for commercial seed and ware potato production throughout the UK and soil inoculum level (DNA of *C. coccodes*) in a 60-g subsample of each soil was determined. A single minituber of cv. Maris Piper was planted in each soil and plants were grown at 18°C. Watering ceased 93 days after planting and progeny tubers were harvested 14 days later and assessed as previously described.

Experiment 5: crop monitoring

In order to ascertain the levels of seed- and soilborne inocula causing disease in commercial seed and ware potato production, 45 crops were monitored in each of three years (2005–2007). These crops consisted of both seed and ware crops and were selected to have a wide geographical distribution across England and Scotland. Various soil types and approximately 30 potato cultivars were represented over the 3 years and the date of the previous potato crop ranged between 4 and 13 years. Industry partners identified a 4- ha (10-acre) area in each field in which soil samples and harvested progeny tuber samples would be taken and records of field disease made. In each case, a soil sample was taken from the field before planting and assessed for *C. coccodes* contamination as described previously. In addition, seed stocks and progeny tubers were assessed visually for black dot and using real-time PCR for *C. coccodes* contamination before planting and at harvest, respectively.

Full datasets for levels of seed- and soilborne inocula of *C. coccodes* and corresponding disease data were obtained from a total of 122 out of a possible 135 crops over 3 years.

An overview of the factors included in each of the experiments 1, 3 and 4 is given in Table 2.

Statistical analysis

All statistical analyses were carried out using GENSTAT 11th edition (VSN International Ltd). Lines were fitted to data plotted on a log (DNA), linear (percentage disease) scale using simple linear regression in Figs 1, 4, 5 and 6. The Mann–Whitney test was used to statistically analyse the differences in the amount of detectable *C. coccodes* DNA on seed tubers with different disease-symptom severities. The effect of seed inoculum level on progeny tuber disease in individual field trials (experiment 2: d.f. = 3) was analysed using ANOVA. The effect of seed inoculum level, temperature and moisture levels in controlled-environment experiment 1 (d.f. = 4), and the effect of soil inoculum level and temperature in experiment 3 (d.f. = 3) were analysed using ANOVA.

Table 2 Overview of treatments in controlled-environment experiments 1, 3 and 4

Expt. no.	Inoculum source and level	Cultivar	Temperature (°C)	Moisture regime[a]
1	Seed tubers: four levels naturally contaminated	Maris Piper	18, 22	damp, dry
3	Soil: five levels artificially infested	Maris Piper, Saxon	18, 22	damp
4	Soil: 32 levels naturally contaminated	Maris Piper	18	damp

[a]In damp treatment, constant dampness of compost was maintained; dry treatment received half the water of the damp treatment.

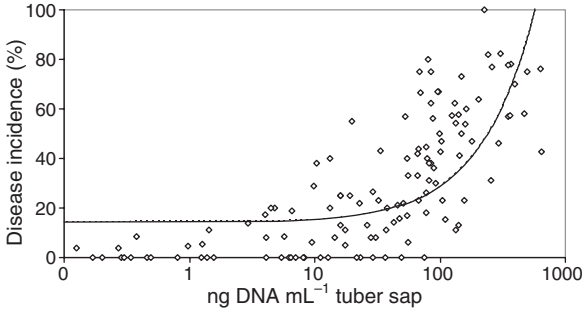

Figure 1 Relationship between black dot visual symptoms, expressed as percentage disease incidence, and the level of detectable *Colletotrichum coccodes* DNA on progeny potato tubers. Line fitted by linear regression (variance accounted for = 46%; $P < 0{\cdot}01$).

Results

Relationship between visual disease assessment and *C. coccodes* DNA levels on tubers

Throughout these studies, visual assessments of disease symptoms and the level of *C. coccodes* DNA were determined on progeny tubers in tandem. An analysis of data from experiments 1, 3 and 4 showed that there was a highly significant ($P < 0{\cdot}01$) relationship between disease incidence and detectable DNA (Fig. 1). As the level of detectable DNA increased above 10 ng DNA mL^{-1} tuber sap, so did the incidence of black dot on tubers. At levels of DNA <10 ng mL^{-1} tuber sap, the corresponding level of disease incidence was generally low, i.e. ranging from no visual disease symptoms up to 20% incidence. The variation in disease incidence observed at levels of between 10 and 100 ng DNA mL^{-1} tuber sap may be attributed to some tubers being infected with *C. coccodes* but not expressing black dot symptoms.

Relationship between seedborne inoculum and black dot development

Contamination of seed tubers with *C. coccodes* (ng DNA mL^{-1} tuber sap) was related to visual disease category (Table 3). However, significant differences in contamination were only observed when the two lower visual disease categories (no symptoms and <5% surface area) were compared with the two higher visual disease categories (5–20% and >20% surface area). As the tubers in each visual disease category were selected from the same stock, the detection of *C. coccodes* DNA on symptomless tubers was probably caused by contamination originating from diseased tubers, particularly if the stock selected had overall high levels of disease incidence and severity.

Table 3 Amount of *Colletotrichum coccodes* DNA (ng DNA mL^{-1} tuber sap) on potato seed tubers categorized according to percentage surface area with black dot symptoms in the seed stock used in both 2005 field trials and controlled-environment experiment 1

	Percentage tuber surface area with black dot symptoms			
	No symptoms	<5%	5–20%	>20%
C. coccodes DNA	137·9 a	148·0 a	253·0 b	344·9 b
Standard error	31·71	16·91	31·91	48·90

Values represent means of 24 individual tubers and values followed by different letters are significantly different (Mann–Whitney $P < 0{\cdot}05$).

In all four field trials (experiment 2), the level of seed inoculum did not significantly affect either the incidence or severity of disease on progeny tubers, or the percentage of progeny tubers deemed unmarketable (i.e. with >10% surface area diseased) (Fig. 2.) At the Scottish field-trial sites, disease incidence (<20%), average disease severity (<2%) and percentage unmarketable tubers (<2%) were low at all seed inoculum levels in both years. At the English field trial sites, although disease incidence was higher (*c.* 60%) than at the Scottish sites, average disease severity was relatively low (<4%) and percentage unmarketable tubers generally low (<10%). Soil inoculum levels at all four field trial sites were less than 40 pg DNA g^{-1} soil (Table 2) and were particularly low (3–5 pg DNA g^{-1} soil) at both sites tested in 2004.

When the same seed stock used in the 2005 field trials was planted in non-contaminated compost and grown under controlled environmental conditions considered conducive to black dot development (experiment 1), seed inoculum level did not significantly affect disease incidence or severity on the progeny tubers (Table 4). The incidence of disease on progeny tubers was approximately 25% higher than at the Scottish field trial site in 2005, but lower than that at the English field trial site in that year. The severity of disease was higher under controlled environmental conditions than at both the field trial sites, but was still relatively low (<4·3%).

Warm (22°C, as opposed to 18°C) and damp conditions significantly increased the incidence and severity of disease on cv. Maris Piper progeny tubers in experiment 1 ($P < 0{\cdot}05$) (Fig. 3). However, in experiment 3, although the incidence and severity of disease were greater at the higher temperature under moist conditions, this difference was not significant.

Results from monitoring of 122 commercial crops (experiment 5) revealed that in all years, a high proportion of seed stocks were contaminated with *C. coccodes* (over 75%). Moreover, in 19% of the seed stocks tested each year, real-time PCR assays detected *C. coccodes* contamination on seed stocks that did not have visual symptoms of disease. There was, however, no relationship between the level of seed inoculum and disease incidence or severity on the progeny tubers (data not shown). Within the monitored crops there were 27 instances where no soil inoculum was detected. Of these, 23 crops were planted with seed having detectable levels of *C. coccodes* contamination and disease occurred in

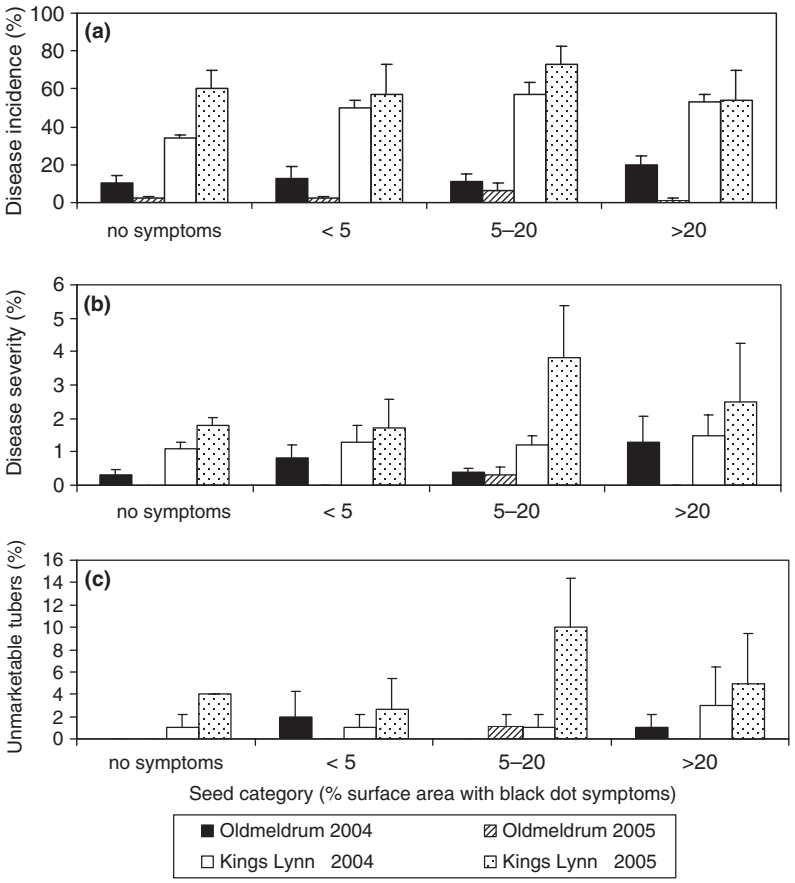

Figure 2 Effect of potato seed-tuber inoculum level (according to percentage surface area with black dot (*Colletotrichum coccodes*) symptoms) on (a) disease incidence, (b) disease severity (mean percentage surface area showing symptoms) and (c) percentage unmarketable tubers in progeny tubers harvested from field trials with low levels of soil inoculum. Values are means of four randomized plots within each trial. Vertical bars represent standard error of means.

Table 4 Incidence and severity of black dot (*Colletotrichum coccodes*) on progeny tubers when infested seed tubers (categorized according to percentage surface area with black dot symptoms) were grown in uncontaminated compost under controlled-environment conditions (experiment 1)

	Percentage tuber surface area with disease symptoms			
	No symptoms	<5%	5–20%	>20%
Progeny				
Black dot disease incidence (%)	25·8 (5·7)	25·4 (4·5)	27·5 (6·1)	22·9 (4·7)
Black dot disease severity (%)	3·4 (1·2)	3·6 (1·0)	4·3 (1·2)	3·0 (1·0)

Values are means of two temperatures and two water regimes (standard errors of means in parentheses).

approximately a third of these crops. However, in all but one case, incidence of disease in the crop was less than 25% (data not shown).

Relationship between soilborne inoculum and disease

In experiment 3 there was a significant increase in disease incidence on progeny tubers related to increasing levels of soil infestation (Fig. 4). The highest infestation level used (9958 CFU g^{-1} soil) resulted in disease incidences of 50% and 23% on Maris Piper and Saxon progeny tubers, respectively, illustrating the difference in cultivar disease resistance rating.

Similarly, when minitubers were grown in naturally contaminated field soils (experiment 4) under controlled environmental conditions, the incidence of disease significantly increased as the level of detectable *C. coccodes* inoculum in the soil increased (Fig. 5). Plant growth in this experiment was not as vigorous as in experiments 1 and 3, mainly as a result of some soils becoming compacted during various handling procedures. This may account for the lower levels of disease incidence in some soils than expected.

Following direct extraction of DNA from 122 field soils as part of the crop-monitoring experiment (experiment 5), *C. coccodes* DNA was detected at levels ranging

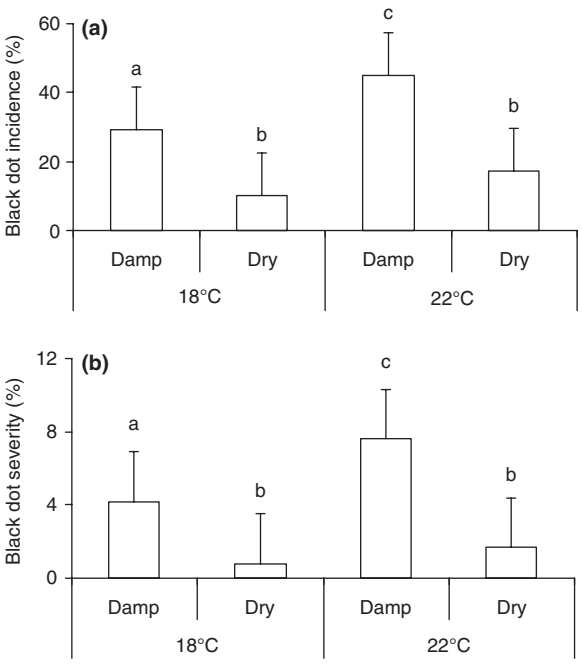

Figure 3 Effect of temperature and water regime in experiment 1 on (a) incidence and (b) severity of black dot (*Colletotrichum coccodes*) disease symptoms on progeny potato tubers grown in compost under controlled environmental conditions. Values are mean of four seed-tuber inoculum levels. Vertical bars represent least significant differences ($P = 0{\cdot}05$): treatments with different letters are significantly different: ANOVA ($P < 0{\cdot}05$).

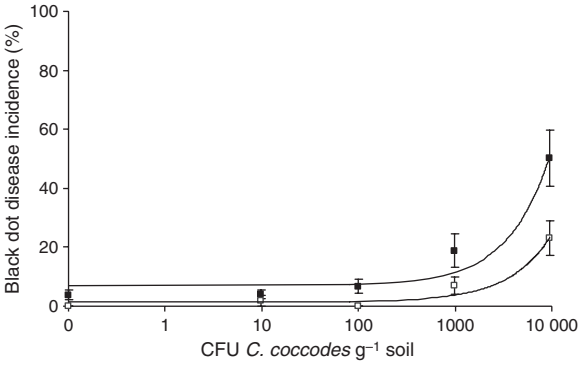

Figure 4 Incidence of black dot (*Colletotrichum coccodes*) on progeny potato tubers of cvs Maris Piper (filled symbols) and Saxon (open symbols) grown under controlled environmental conditions (experiment 3) in artificially infested soil. Values are means of two temperatures. Vertical bars represent standard errors of means. Lines fitted by regression; Maris Piper $P < 0{\cdot}01$, variance accounted for = 47%; Saxon $P < 0{\cdot}01$, variance accounted for = 38%.

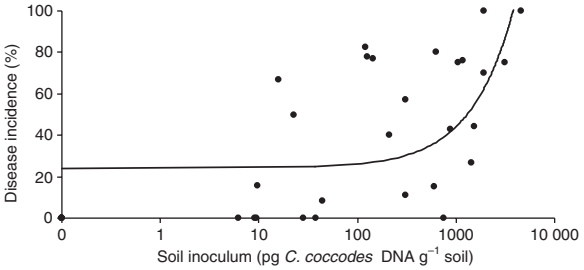

Figure 5 Incidence of disease on progeny potato tubers grown under controlled-environment conditions in field soils which were naturally contaminated with different levels of soil inoculum of *Colletotrichum coccodes* (experiment 4). Line fitted by regression; $P < 0{\cdot}01$, variance accounted for = 31%.

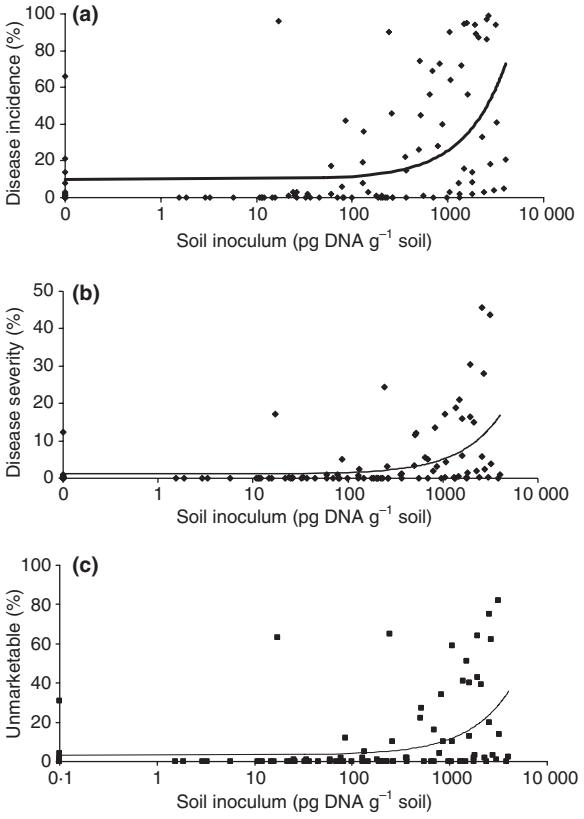

Figure 6 Relationship between soil inoculum of *Colletotrichum coccodes* and (a) incidence and (b) severity of black dot symptoms on progeny potato tubers and (c) percentage unmarketable tubers in 122 commercial seed and ware crops. Lines fitted by regression, $P < 0{\cdot}01$, variance accounted for = 23%, 20% and 17% for disease incidence, severity and unmarketable tubers, respectively.

from zero (undetectable) to over 7100 pg DNA g^{-1} soil (Fig. 6). Across the 3 years of monitoring a high percentage of soils (74%) were contaminated with *C. coccodes*.

In the crop-monitoring trials (experiment 5), the level of soil infestation (pg DNA g^{-1} soil) was found to have a significant effect on the incidence and severity of disease and percentage unmarketable tubers in the progeny crop (Fig. 6). At levels of soil infestation below 100 pg DNA g^{-1} soil, very few crops had more than 20% incidence of black dot and increasing soil infestation levels resulted in an increase in the incidence of black dot

(Fig. 6a). The severity of black dot on progeny tubers also increased as the level of soil infestation increased (Fig. 6b). In contrast with seedborne inoculum, soilborne inoculum had the potential to result in high levels of both disease incidence and severity and percentage unmarketable tubers (Fig. 6c). The percentage variation accounted for by the linear regression (fitted to Figs 6a,b and c) was quite low because disease resulting from high inoculum levels was very variable. For example, at soil inoculum levels above 1000 pg DNA g^{-1} soil, whilst 96% of crops had some level of black dot, the incidence of disease ranged from 2% to 99%. The monitored crops encompassed many crop variables, for example varieties with different black dot resistance ratings and crops grown with a range of crop durations, irrigation and the use of azoxystrobin (Amistar, Syngenta Crop Protection), all factors which may significantly influence black dot development.

It was attempted to set arbitrary threshold levels for soilborne inoculum, relating the level of soil inoculum to a category of disease risk. The percentage of crops with disease, and also those with more than a 20% incidence of disease, increased with increasing disease-risk category (Table 5). At soil infestation levels below 100 pg DNA *C. coccodes* g^{-1} soil, 7% of commercial crops had an incidence of black dot greater than 20%, increasing to 40% and 57% of crops at levels of 100–1000 pg g^{-1} and >1000 pg g^{-1} soil, respectively. However, interpretation of risks based on such thresholds must, in the future, be informed by knowledge of agronomic variables and potential control strategies.

Discussion

Diagnostic testing of soil and tubers for *C. coccodes* has provided for the first time an accurate quantification of inoculum from which results of trials and experiments can be interpreted.

Overall, seedborne inoculum was found to be relatively less important than soilborne inoculum in causing black dot on progeny tubers at harvest. The incidence and severity of black dot at harvest were very low in trials where seed was the main source of inoculum, irrespective of the level of seedborne inoculum visibly present at planting. This is in agreement with the previous findings of Denner *et al.* (1998) and Nitzan *et al.* (2008) amongst others. The higher incidence of disease at the English trial sites in both years cannot be explained by differences in soil inoculum levels. Other factors such as crop duration, temperature, moisture and soil type may possibly have contributed to these differences in disease incidence. In trials where both seed- and soilborne inocula were present, the presence of seed inoculum increased the level of black dot on progeny tubers. Thus, whilst seed inoculum may be relatively less important in contributing to final disease levels, it may enhance the effect of soil inoculum. In addition, infected seed tubers may introduce black dot into previously uncontaminated land and therefore, where possible, seed infection should be kept to a minimum. Tuber diagnostic tests carried out on seed stocks graded into different levels of contamination showed that symptomless tubers could still be infected and that only extreme inoculum categories could be assigned by visual assessment. In field trials and controlled-environment experiments this finding was confirmed when seed with no visible black dot present and visibly infected seed resulted in similar levels of disease on progeny tubers. The inaccuracy of using visual assessments may also explain the results of Read & Hide (1988), who found no difference in the amount of tuber progeny disease resulting from planting seed with >5% or >30% surface area affected with black dot compared with disease-free seed in trials conducted in 1985 and 1986 respectively. In the latter case the disease-free seed was selected from the same stock as the diseased seed and the confounding effect of symptomless contamination could therefore not be ruled out. Similarly, the results of later work by the same authors (Read & Hide, 1995) also showed that disease was found on the progeny of plants grown from disease free seed and significant effects between seed disease categories were only noted at the extremes.

Although the general findings regarding the importance of seedborne inoculum are in agreement with previous studies, this work is the first that has allowed an accurate assessment of seedborne inoculum levels. Previous authors demonstrated that there was no significant difference in progeny disease caused by the planting of seed carrying different levels of inoculum (e.g. Read & Hide, 1988; Denner *et al.*, 1998), but these earlier estimations of seed inoculum level were made by visual assessments only. It has now been shown definitively that measuring such differences in inoculum using visual assessments is generally valid, but is not accurate enough to separate seed into inoculum categories and cannot account for symptomless sources of contamination that may confound results.

Where a seed stock exhibits black dot, grading out infected tubers will therefore not necessarily reduce the risk of black dot where soilborne inoculum is known not to exist. A seed stock with undetectable levels of inoculum, as determined by real-time PCR, could potentially reduce this risk, by identifying stocks with symptomless infections.

Table 5 Percentage of crops of potato tubers with black dot (*Colletotrichum coccodes*) and percentage with an incidence of black dot greater than 20% when a range of seed and ware crops were grown in soils allocated to different disease-risk categories (experiment 5)

	Disease-risk category (pg DNA g^{-1} soil)		
	Low	Medium	High
	<100	100–1000	>1000
Percentage crops with disease	35	63	96
Percentage crops with disease incidence >20%	7	40	57
Number of crops in category	55	30	28

A stronger relationship was found between the level of *C. coccodes* soil inoculum (measured as pg DNA g^{-1} soil) and black dot on progeny tubers, and because seed inoculum levels were also measured in all trials the contribution of each source of inoculum could be assessed. Trials under controlled environmental conditions showed that there was a trend for increasing tuber disease with increasing soil inoculum concentration in the absence of other factors such as soil type, crop duration and agronomic practice. This relationship was altered by host resistance, as would be expected, with the more resistant cv. Saxon having significantly less disease at high inoculum levels than the more susceptible cv. Maris Piper. In addition, there was an effect of moisture and temperature on disease development, with more disease occurring at 22°C than at 18°C, and under damp conditions in experiments 1 and 3, although this relationship was only significant in experiment 1. Accurate soil moisture measurements were not taken in these experiments, but previous work by Adams *et al.* (1987) showed that black dot is increased by irrigation and this area should be investigated in more detail in the context of soil inoculum levels and other disease-management practices.

Reasons for the low incidence of disease on progeny tubers when the inoculum originates from seed- as opposed to soilborne inoculum are difficult to explain. Andrivon *et al.* (1998) observed that symptoms resulting from seedborne inoculum developed first on roots, then on stolons and finally on stems and daughter tubers. Root and stolon infections appeared at, or shortly after, emergence of these plant organs, whereas stem infections developed only when inoculum levels on below-ground organs were high (7–10 weeks after inoculation, depending on cultivar).

There has been little work to explain the aetiology of black dot caused by soilborne inoculum. It remains unreported whether microsclerotia of *C. coccodes* are distributed evenly in the soil or are present within foci. However, it can be speculated that where microsclerotia are adjacent to potato tissue (stems, stolons, mother tubers, roots or progeny tubers) they may germinate and infect the nearest host tissue. The extent to which the pathogen can grow through the soil to reach the host is also uncertain. However, higher levels of soil inoculum presumably increase the likelihood of contact between the host and pathogen. The greater relative importance of soilborne inoculum could possibly therefore be the result of increased opportunity for infections to occur, as compared with seedborne inoculum, which is contained within a small area around the mother tuber. Further work is required to elucidate the reasons for this difference in importance of inoculum sources.

It is difficult to compare the results relating to soil inoculum levels with those of Nitzan *et al.* (2008) as the inoculum levels are not directly comparable and different disease measurements were made (tuber disease in the present study, as opposed to foliar symptoms and measurements of sclerotial density on roots and stems in the work of Nitzan *et al.*, 2008). It is possible that had higher levels of soil inoculum been used in the present study then an upper threshold for disease development might have been observed. However, the highest level of *C. coccodes* soil inoculum detected in any of the 122 commercial crops tested was 7100 pg DNA g^{-1} soil. This is within the same range of inoculum concentrations tested in the artificially inoculated soils, where the highest soil inoculum concentration equated to approximately 6000 pg DNA g^{-1} soil. Therefore, it can be assumed that the tuber disease measured was probably representative of that caused by naturally occurring soilborne inoculum in the UK. The trend for increasing disease on tubers as related to increasing soil inoculum level under controlled conditions was corroborated by quantitative measurements of naturally occurring soil inoculum in commercial crops.

Colletotrichum coccodes soil DNA concentrations of 100 and 1000 pg DNA g^{-1} soil measured using the tests employed in this work have been chosen to provide guidance on medium and high risk categories, respectively, for black dot development. In a monitoring exercise where black dot soil contamination was determined in 112 commercial field soils, around 50% of fields were found to exceed the lower category of 100 pg DNA g^{-1} soil. The adoption of the two categories was supported by results from controlled-environment experiments, a disease-monitoring exercise and field trials carried out across 20 sites in 2007 (results not shown).

The quantification of *C. coccodes* inoculum has allowed the relative contributions of different inoculum sources in causing disease to be interpreted more accurately and disease-risk categories to be set based on these findings. Where a number of fields are tested for *C. coccodes* contamination, crop management decisions can be taken. At the most basic level, fields with the highest degree of soil contamination can be avoided, or decisions regarding cultivars to be planted can be made. This may be particularly useful on rented land where the cropping history may not be known. On land which is not contaminated or where there is a low risk, planting of seed infected with *C. coccodes* should be avoided to prevent further soil contamination. Other factors that may affect black dot development, for example, crop duration, crop agronomy, irrigation and storage, should be investigated as part of an integrated disease management system in addition to the effect of soil inoculum level and its interaction with host resistance.

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