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DETECTION OF SEED TO SEED TRANSMISSION OF *BOTRYTIS CINEREA* IN LETTUCE

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A B S T R A C T

Botrytis cinerea is pathogenic to many crops including lettuce. The aim of this study was to test whether *B. cinerea* contaminated seed could give rise to infected plants and whether infected plants could produce infected seed as a means of confirming the endophytic life cycle. *B. cinerea* was detected in seed and within plants both by isolation on Botrytis selective medium (BSM) and by qPCR, with and without prior surface sterilization. *B. cinerea* was detected more frequently by qPCR ($P<0.001$) and isolation on BSM in cv. Diana and cv. Tom Thumb respectively. Within growing plant stems qPCR detected more *B. cinerea* while in roots and leaves more of the pathogen was recovered on BSM. The detection of *B. cinerea* in lettuce seedlings grown from infected seed in isolation is evidence of seed to seedling transmission while detection of infection in seeds harvested from plants which were grown in the field and flower/bud inoculated proves that there was seedling to seed transmission.

Keywords: qPCR, seedborne, systemic, Taqman assay, transmission

INTRODUCTION

Lettuce is an important vegetable crop which is consumed in many parts of the world. Its production is however hampered by many fungal diseases which reduce the quantity and quality of the produce. Among the numerous fungal diseases which affect lettuce crops, particularly in the temperate regions, is grey mould caused by *Botrytis cinerea*. There are some reports on seed to seedling transmission of *B. cinerea,* leading to systemic infection of the growing plant. For instance, Author *et al*. (2010) reported that *B. cinerea* can be transmitted from seed to seedlings in lettuce. However seeds may not be directly infected from a systemic infection but rather via flower infection originating from spores (Van Kan*et al*., 2014).

Seed borne infection in Primula led to a systemic seedling infection in the absence of external inoculum (Barnes and Shaw, 2003). This confirms Harold *et al*. (1997) report that *B. cinerea* was transmitted from seed to seedlings in linseed (*Linumusitatissimum*) The isolation of *B. cinerea* from diseased seedlings and seed samples of Chickpea by Burgess *et al*. (1997) was confirmed by Author *et al*. (2010) who recovered *B. cinerea*from internal tissues of plants grown in spore-free air flows from surface sterilized seed of Lettuce cv. All the Year Round and Tom Thumb. However, it is not certain if seedling infection by *B. cinerea* can be transmitted directly to the seed produced on an infected plant. This study therefore sought to test direct internal seed to seed transmission of *B. cinerea,* by detecting it in plants grown from infected seed and in seed harvested from infected plants. A secondary aim was to test the relative efficiency of detection of *B. cinerea* by isolation on Botrytis Selective Medium (BSM) and by Quantitative Polymerase Chain Reaction (qPCR). The findings will further clarify the endophytic life cycle and aid in disease control.

MATERIALS AND METHODS

Sampling design

Field experiment

Nursing and transplanting of lettuce seedlings: In 2005, seeds of two commercial lettuce cultivars, Saladin Supreme and Diana (Tozers, UK) and Tom Thumb (Johnsons Seeds, Newmarket, Suffolk, UK) were nursed in soil-based compost (John Innes) contained in plug

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trays, with or without sterilization. Prior to sowing, seeds were sterilized with 0.1% Sodium hypochlorite contained in Domestos, commercial bleach with a surfactant (Clarkson and Moule, 1998) for 5 min, rinsed in two changes of sterile distilled water and dried for 30 min. Watering was done daily until 6 weeks, when seedlings were transferred to a cold frame for a week to acclimatize before transplanting in the experimental grounds of the University of Reading, UK. Six treatments consisting of plants from three cultivars of lettuce above, grown from sterilized or unsterilized seed, were arranged in a randomized complete block design with four replications per treatment. Plants were 25 cm apart with 40 cm between rows.

Inoculation procedure: Flowers and unopened buds of sixty plants, covered with transparent polyethylene bags, were inoculated 3 months after planting, with dry spores (Barnes, 2002) of a root isolate of *B. cinerea* (ES27) harvested from a two-week old culture, applied through a needle (25mm 63/100 23GX1) attached to a 10 ml syringe (BD Plastipak, UK). The inoculated flowers and buds remained covered for 24 h to facilitate spore germination. This procedure was repeated when more flowers emerged.

Isolation experiment: This experiment was first conducted in 2005 and then repeated in 2006 with seeds of cv. Tom Thumb (Johnsons Seeds, New market, Suffolk, UK). Infected seeds from the field experiment in 2005, which were harvested from asymptomatic plants grown from unsterilized seed and inoculated with ES27, a root isolate and the original seed (uninfected) purchased in 2005 and stored at 5 °C were used. A 2^2 factorial design with trays in the isolation plant propagator (Burkard Manufacturing Co., UK) as blocks each containing 7 replicates randomly arranged was used. There were 4 treatments consisting of infected or uninfected seeds which were either sterilized or unsterilized. Based on germination tests, appropriate numbers of seeds were sown in soil-based compost (John Innes) contained in 6" pots. At 6 weeks after planting, single plants were harvested from each pot and sectioned into root, stem and leaf.

Detection of *B. cinerea* in seed and plant parts: The plating test and a Quantitative Polymerase Chain Reaction (qPCR) were used to detect *B. cinerea* in seeds from inoculated and uninoculated plants grown in the field and in tissue samples from plants grown in a spore-free air flow in an isolation plant propagator from surface sterilized seed with internal infection (Author *et al*., 2010). Seeds of cv. Tom Thumb and cv. Diana (Unwins Seeds Ltd, Cambridge, UK) harvested from plants originally grown in a glasshouse were used.

Plating test: Fifty seeds were plated harvested from plant/plants with each factorial combination of cultivar, parent seed sterilization and bud/flower inoculation. *B. cinerea* was isolated from equal numbers of seeds on Botrytis Selective Medium (Edwards and Seddon, 2001). The seed samples were obtained from inoculated plants of cv. Tom Thumb and Diana, grown in the field from sterilized seed. All seeds were surface sterilized as described earlier.

The root, stem and leaf sections from the isolation experiment were cut into 3 one cm-long pieces. A piece each of the root, stem and leaf from a plant were surface sterilized, placed in the same BSM plate, incubated and scored.

Quantitative Polymerase Chain Reaction (qPCR)

DNA extraction from seed and plant parts: DNA was extracted from single and bulk seeds harvested from lettuce plants in the field experiment described above. For bulk extraction, 10 seeds from each sample were used while extractions were made from 10 individual seeds per sample in the case of single seeds. Seeds were ground with liquid nitrogen in eppendorf tubes using a plastic rod. To the powder, 0.6 ml of extraction buffer was added and the mixture incubated for 30 min at 70 °C. The extraction buffer was made up of 100 ml of a 10% solution of SDS, 14.6 g of NaCl, 200 ml of 1M stock (pH 8) of TrisHCl, 100 ml of 0.5M stock of EDTA (diSodium), 20 g of PVP, 2 g of Phenanthroline, and 4.75 µl of ß-mercaptoethanol which was added to 100 ml of the stock solution immediately before use. PVP was dissolved separately by heating in a water bath at 60 °C with stirring and then added to the buffer. About 1 ml of methanol was added to the stock solution to facilitate the complete dissolution of phenanthroline. After incubation, 0.3 ml of 5M NH4Acetate (28.905 g in 50 ml) was added to each eppendorf. The mixture was incubated on ice for 30 min before centrifuging for 10 min at 13000 rpm. The supernatant was decanted and the pellet discarded. All the supernatant was transferred to a new tube with 0.3 ml of -20 °C isopropanol on ice and mixed by vortexing before centrifuging at 13000 rpm for 10 min. The supernatant was poured off and 0.2 ml of ice cold 70 % ethanol added before centrifuging at 13000 rpm for 5 min. The supernatant was poured off

and the pellet dried briefly at 13000 rpm for 2 min. The pellet was resuspended in 50 µl of water.

DNA was extracted from the root, stem and leaf sections of cv. Tom Thumb plants, grown under a spore-free airflow in a plant propagator, with the DNeasy Plant Mini kit (Qiagen, West Sussex, UK). All DNA samples were stored at -20 °C until required for qPCR.

Preliminary Tests: A standard curve (10 ng µl^{-1}, 1 ng µl^{-1}, 0.1 ng µl^{-1}, 0.01 ng µl^{-1}, 0.001 ng µl^{-1} and 0.0001 ng µl^{-1} with two water controls) was prepared with DNA of single and bulk seeds which were previously quantified by agarose gel electrophoresis, spectrophotometry and Pico-Green assay. A master mix containing 12.5 ul qPCR master mix, 1 µl each of forward and reverse primers, 0.025 µl of probe, 5 ul of template and 5 µl of water per reaction was prepared and 20 µl aliquoted into each well of the plate. To each well 5 µl of the appropriate DNA sample was added. The plate was covered and the contents of the wells mixed by centrifuging before running the qPCR in an AB7700 machine (Abgene, UK). Another run of the qPCR was carried out with DNA extracted from seed samples with known proportions of infection estimated from the results of seed plating tests.

Primer ratio: Two standardized extracts of DNA were used. These were DNA samples of *Botrytis cinerea* which were quantified by Pico-Green assay (Invitrogen, UK) and their concentrations adjusted to 10 ng µl^{-1}: the standard, 1 ng µl^{-1} and 0.1 ng µl^{-1} with water as a control. Three concentrations of each DNA sample and water were used. Apart from the original concentration of 10 ng µl^{-1}, each sample was diluted twice by 1 in 10 to 1 ng ul^{-1} and 0.1 ng µl^{-1} with water as the control. Five master mixes containing the following ratios of the forward and reverse primers respectively were prepared: 1:1, 5:3, 3:5, 5/3:1, 1:5/3. Each reaction was made up of 12.5 µl qPCR master mix (Abgene, UK), the appropriate proportions of the forward and reverse primers (Sigma-Genosys, UK) depending on the ratio, for example 1 µl each for the ratio 1:1, 0.05 µl probe (Sigma-Genosys, UK), 5 µl water and 5 µl template. The samples were loaded in a 96-well plate before running the qPCR in an AB7700 machine (Abgene, UK). There was no noticeable difference between the primer ratios so 1:1 was used in subsequent reactions.

Sensitivity and Consistency: The total reaction volume was 25 µl, made up of 12.5 µl qPCR master mix, 1 µl each of forward and reverse primers, 0.05 µl of probe, 5 µl of water and 5 µl of template. Probe sensitivity, interference by plant DNA and isolate variability were tested. Interference by plant DNA was tested with DNA from lettuce seeds, corn and wheat flours which was extracted twice from each material with the DNeasy Plant Mini Kit (Qiagen, West Sussex, UK). The DNA samples were quantified by spectrophotometry and their concentrations adjusted to 10 ng/µl. To test sensitivity and probe concentration, probe concentrations of 200 nM, 100 nM and 50 nM were used with six different concentrations of the target *B. cinerea* DNA which were 10ng µl^{-1}, 1 ng µl^{-1}, 0.1 ng µl^{-1}, 0.01 ng µl^{-1}, 0.001 ng µl^{-1} and 0.0001 ng µl^{-1}, obtained by making serial 1 in 10 dilutions of the starting material. There were two water controls for each probe concentration. To determine the effect of plant DNA, six serial 1 in 10 dilutions of each plant DNA sample with an initial concentration of 5 ng plus 5 ng of *B. cinerea* DNA were made and then run with three different probe concentrations of 200 nM, 100 nM and 50 nM. To test isolate variability, DNA samples of *B. cinerea* isolates from root, stem and leaf of an uninoculated plant of cv. All The Year Round (Unwin's Seeds Ltd, Cambridge, UK) grown in a glasshouse from sterilized seeds were used. Six serial 1 in 10 dilutions of these samples were made and run with a probe concentration of 100 nM. After sealing the plate, it was centrifuged for 5 min to mix the contents of each well before running the qPCR.

Detection of *B. cinerea* DNA in seed and plant DNA: The forward (GTTACTTGACATGCTCTGCCATT) and reverse (CACGGCTACAGAAAGTTAGTTTCTACAA) sequences were used with the probe (ATTTTGGCAGATTGATTACAGGGCAAACTTACA-FAM/TAMRA) to detect the intergenic spacer (IGS) region of the nuclear ribosomal DNA of *B. cinerea* (Suarez *et al*., 2005) from seeds and plant parts. The *Botrytis cinerea*Taqman assay was possible because the Central Science Laboratories, UK made available the probe.

RESULTS

Preliminary tests: The ratio 1:1 for the forward and reverse primers was chosen for convenience because there was no significant difference (Anova: P=0.99) between the various ratios. Comparisons were made between different host plants to determine if their DNA would interfere with the sensitivity of the probe. A test with three probe concentrations viz. 50 nM, 100 nM and 200 nM in corn, lettuce and wheat DNA backgrounds showed that the sensitivity of the probe was not affected (P=1.0). All the probe concentrations yielded positive

results with pathogen DNA as low as 0.1 ng µl^{-1} in all backgrounds. The 200 nM and 50 nM yielded negative results at a pathogen DNA concentration of 0.01 ng µl^{-1} with lettuce DNA as background so the probe concentration 100 nM was used as the standard.

Detection of relative infection levels in seeds and plant parts: Generally inoculated seed had more infection than uninoculated ones but unsterilized and inoculated seed had more infection than sterilized and inoculated seed. Flower/bud inoculation of plants grown in the field resulted in seed infection, which was detected by both BSM and qPCR (Table 1). However, qPCR detected more internal infection (75%) in cv. Diana than plating on BSM (35%), but in cv. Tom Thumb BSM plating detected more (65%) than qPCR (30%) (Interaction P =0.01, Ftest with GLM using a binomial error). The main effects of sterilization and inoculation were significant at P = 002 and P<0.001 respectively. All these seed came from single plants, so there is no independent measure of plant-plant variation within a treatment and the residual is that from interactions between the factors. Based on the residual, both the effect of bud inoculation and sterilizing the original seed have effects in the direction expected if internal infection is transmitted to seed, and both are very significant (P <0.01). Bud inoculation increases infection rate by a factor of about 3(F=20.7, df 1, 8, P=0.002); sterilization of the original seed decreases infection rate by a factor of about 0.05 (F=14.4, df 1, 8, P=0.005) (Table 2).

Seedlings of cv. Tom Thumb grown from infected seed under a spore-free air flow in an isolation plant propagator had infection in their roots, stems and leaves which was detected by BSM and qPCR (Table 3). Generally, roots had the highest infection followed by stems and then leaves. More *B. cinerea* was recovered from stems by qPCR (32%) than BSM (18%). However for roots and leaves, BSM recovered more *B. cinerea* than the qPCR. In leaves *B. cinerea* was recovered by BSM when qPCR could not detect its presence. Detection in roots, stems and leaves suggests that *B. cinerea* is systemic and was transmitted from infected seed.

Table 1: Number of individual surface sterilized lettuce seed collected from a field experiment testing positive for *Botrytis cinerea* presence by two different methods.

Variety	Original seed Sterilization	Bud Inoculated	*B. cinerea* recovered by BSM (n=50)	qPCR (n=10)
Diana	Sterilized	Inoculated	21	8
	Sterilized	Uninoculated	9	5
	Unsterilized	Inoculated	24	10
	Unsterilized	Uninoculated	16	7
Tom Thumb	Sterilized	Inoculated	28	3
	Sterilized	Uninoculated	-	-
	Unsterilized	Inoculated	42	4
	Unsterilized	Uninoculated	27	2

SD of mean = 2.3

Table 2: Analysis of deviance showing effects of three main factors; method of detection, cultivar and bud inoculation on the incidence of *B. cinerea* in lettuce plants grown in the field

Change	d.f.	Deviance	Mean Deviance	Deviance ratio	Approx. F pr.
+ Method	1	1.4956	1.4956	1.84	0.212
+ Variety	1	12.3094	12.3094	15.14	0.005
+ Method.Variety	1	32.8232	32.8232	40.37	<.001
+ Bud_inoc	1	16.8480	16.8480	20.72	0.002
+ Original_ster	1	11.6914	11.6914	14.38	0.005
Residual	8	6.5052	0.8131		
Total	13	81.6729	6.2825		

Table 3: Relative infection of plant parts of Tom Thumb grown in an isolation plant propagator

Part	Total Sampled	% *B. Cinerea infection*	
		qPCR	BSM
Root	30	27	37
Stem	22	32	18
Leaf	30	0	23

SD of mean = 2.18

DISCUSSION

In this study, *B. cinerea* was detected in seedlings grown from infected and originally uninfected lettuce seeds. This confirms the isolation of *Botrytis allii* from onion seedlings grown from infected seed (Stewart and Franicevic, 1994). The detection of infection in originally uninfected seed could be attributed to airborne spores. Higher infection in unsterilized/inoculated seed could have resulted from a combination of surface contamination which was removed from sterilized seed and internal infection. This is supported by Maude and Presly (1977a) report that treatment with Chloros (3% free chlorine) reduced but did not eliminate *B. cinerea* and they concluded that the majority of the *B. cinerea* was internal infection rather than surface contamination. Stewart and Franicevic (1994) confirmed the presence of *Botrytis allii* in onion seed through surface sterilization. Sterilized/uninoculated seed may have been infected through exposure to airborne spores as reported by Jarvis (1977) or internal infection which could not be removed through surface sterilization. Flower/bud inoculation of plants grown in the field resulted in seed infection which was detected by both BSM and qPCR but there was no consistency in the amount of *B. cinerea* detected by each method in cv Diana and Tom Thumb. Similarly, there was no consistency in the detection of *B. cinerea* in roots, stems and leaves of infected plants of cv. Tom Thumb which were grown from infected seed under a spore-free air flow in an isolation plant propagator. In other studies, qPCR was consistently better than BSM in detecting *B. cinerea*. For instance, Sanzani *et al*. (2012) reported that qPCR revealed more *B. cinerea* infection in grape berries than the freezing and conventional plating methods, which they attributed to competition against *B. cinerea* by several other microorganisms commonly associated with bunch rot of grape. Chilvers *et al*. (2006) also reported that the real-time PCR assay appeared to be more sensitive than the conventional seed assay on an agar medium in onion. In spite of the different levels of sensitivity, both methods are necessary for various reasons. Although the qPCR is more sensitive, rapid and can detect and quantify fungal DNA regardless of its viability, the conventional plating method is low cost and can be used for the detection and isolation of viable pathogen cells leading to isolates collection that would be used for further analysis, an indication that both methods have complementary advantage for studying latent infection (Wahab and Younis, 2012).

qPCR can detect very minute quantities of DNA in plant material but the level of detection may be limited if there is a sparse distribution of the mycelium. Sparsely distributed mycelium can be detected by plating test because the medium induces the pathogen to grow. In this study when qPCR detected 0% infection in leaves, plating test detected 23%. This suggests that the mycelium may be sparse in leaves making it undetectable by qPCR and easily detectable by plating because *B. cinerea* easily grows in a nutritious medium.

The detection of *B. cinerea* in lettuce seedlings grown from infected seed in isolation is evidence of seed to seedling transmission while detection of infection in seeds harvested from plants grown in the field and flower/bud inoculated, proves that there was seedling to seed transmission. Although all seed infections do not result in seedling infections, it is an important source of inoculum for infection of lettuce by *B. cinerea* (Yahaya and Ahmed, 2015). Since this study has established that seed to seed transmission is possible, infection can be reduced through the application of fungicides such as Benomyl (Benlate) and iprodione (Rovral) which are known to be effective as seed treatments (Yahaya and Ahmed, 2015).

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