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Improved understanding of novel sources of resistance against the light leaf spot pathogen, *Pyrenopeziza brassicae*

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Summary

In this work, the endophytic growth phase of the light leaf spot pathogen *Pyrenopeziza brassicae* in selected lines from a doubled haploid (DH) population of oilseed rape, which is known to segregate for resistance against *P. brassicae*, was characterised using controlled environment (CE) experiments. Fungal staining techniques and pathogen-specific quantitative polymerase chain reactions (qPCR) were used to observe and quantify the pathogen biomass, respectively. The qPCR results showed that the resistant lines contained little *P. brassicae* DNA and there seemed to be little to no change in the amount of DNA over time. In contrast, there was a considerable increase in pathogen DNA in susceptible lines from 0 to 24 days post inoculation (dpi). These results were also reflected in observations made by a fungal staining method. In addition, leaf samples of these DH lines, collected at three different times from winter oilseed rape field experiments, were analysed using qPCR. The resistant lines had a considerably smaller amount of *P. brassicae* DNA in leaf samples collected later in the cropping season than that in susceptible lines

Key words: Oilseed rape, *Pyrenopeziza brassicae*, yield loss, light leaf spot, host resistance, extracellular pathogens

Introduction

The hemibiotrophic fungus *Pyrenopeziza brassicae* is an economically important pathogen of winter oilseed rape in the UK, causing light leaf spot (Boys *et al*., 2007, 2012). Light leaf spot is a polycyclic disease and early infections during autumn and winter are able to kill seedlings, decrease plant vigour and increase susceptibility to frost damage (Fitt *et al.*, 1998; Gilles *et al*., 2001). Light leaf spot on pods causes early maturation and pod shatter leading to further yield losses. Previously, severe disease epidemics have been recorded in Scotland and northern England, where the climatic conditions are favourable for disease development (Figueroa *et al*., 1995). However, according to recent disease survey data, the severity of epidemics has increased progressively across England since 2006 (CropMonitor, 2016). This frequent, widespread occurrence of light leaf spot has made its control a high priority for many oilseed rape growing areas in the UK.

Light leaf spot disease control has mainly relied on the use of fungicides. Fungicide applications can be inefficient due to poor timing and reduced sensitivity to azole fungicides has also been reported (Carter *et al.*, 2014). Therefore, deployment of cultivar resistance against *P. brassicae* is of a greater importance for successful management of this disease. However, there is only a limited amount of information available on the operation of resistance in oilseed rape against *P.*

brassicae. Little is known about the mechanisms of genetic resistance in different commercial oilseed rape cultivars. With recent severe disease epidemics, breakdown of resistance of some cultivars with high resistance ratings has been reported. Therefore, there is a need to improve the understanding about this pathosystem and search for new sources of resistance in different genetic backgrounds. In this study, the endophytic growth phase of the light leaf spot pathogen in selected doubled haploid (DH) lines of oilseed rape derived from a cross between *Brassica rapa* and *B. oleracea* was characterised using *in planta* experiments. These DH lines had been observed to produce different amounts of *P. brassicae* sporulation under controlled environment conditions, three weeks after the conidial inoculation (K Downes, unpublished data) and in field experiments (C S Karandeni Dewage, unpublished data). In this study these DH lines were studied under controlled environment (CE) conditions after artificial inoculation in comparison to winter oilseed rape experiments with natural inoculum in order to assess the development of light leaf spot disease under the two experimental conditions.

Materials and Methods

Plant growth and inoculation in controlled environment (CE) conditions

Eight DH lines (four resistant lines labelled R1-R4 and four susceptible lines labelled S1-S4) and two commercial cultivars susceptible to *P. brassicae* (labelled cv1 and cv2) were grown in a glasshouse until the plants reached growth stage 1,04 (Sylvester-Bradley *et al*., 1985). Plants were point inoculated by placing *c.* 1 cm^2 pieces of Whatman no. 1 filter paper soaked in a *P. brassicae* conidial suspension (10^5 spores mL) on four marked locations on the fourth true leaf. Inoculated plants were transferred into a controlled environment cabinet (A1000, Conviron, Cambridgeshire, UK) at 80% relative humidity and with a 12 h day length with a 210 µe m^2 s^{-1} light intensity. Day and night temperatures were maintained at 16°C and 14°C, respectively. Plants were covered with a polyethylene sheet for 48 h after inoculation to maintain high humidity to facilitate spore germination and infection.

Collection and preparation of leaf samples

Leaf samples from randomly selected plants were collected at 0, 3, 7, 13, and 24 days post inoculation (dpi). Leaf discs of the same size (1.6 cm diameter) were taken from the inoculated fourth true leaf of each plant. The leaf discs used for DNA extraction were inserted into labelled sterile screw-cap tubes and stored at -20°C after freeze drying. The leaf discs used for staining were placed in labelled Petri dishes.

DNA extraction and quantification of P. brassicae *DNA in leaf samples*

Freeze-dried samples were processed in a FastPrep machine (MP Biomedicals, UK) with three sterile metal beads (3 mm diameter) until the leaf discs were ground into a fine powder. DNA was extracted with a DNAMITE DNA extraction kit according to the manufacturer's protocol (DNAMITE Plant Kit, Microzone Ltd, UK). The DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK) and samples were adjusted to a final concentration of 20 ηg µL with sterile distilled water for qPCR analysis.

The amount of *P. brassicae* in 50 ng of DNA from each leaf sample was quantified using *P. brassicae* diagnostic primers PbITSF and PbITSR (Karolewski *et al*., 2006) using an Mx3005 qPCR instrument (Agilent technologies, UK). qPCR reaction mixture was prepared by adding 10 µL Brilliant III ultra-fast SYBR Green qPCR mix, 6.3 µL sterile distilled water, 0.6 µL (10 µM) PbITSF, 0.6 µL (10 µM) PbITSR and 2.5 µL (20 ηg µL) template DNA. Five standards were prepared using *P. brassicae* DNA ranging from 10000 pg to 1 pg to plot a standard graph for *P. brassicae* DNA quantification. Template DNA replaced with sterile distilled water was the negative control and 1 ηg of *P. brassicae* DNA was the positive control.

No-template controls, five standards, positive control and plant sample DNA were added in duplicate for each run, in 96 mL × 0.2 mL PCR plates (ABgene) covered with cap strips and the

PCR conditions were set for an initial denaturation temperature of 95°C for 2 min followed by 50 cycles of 15 s at 95°C, 45 s at 58°C, 45 s at 72°C and a data collection step of 15 s at 84°C.The final step of the qPCR consisted of a melting curve with cycling parameters of 95°C for 1 min, 58°C for 30 s and 95°C for 30 s. The amount of *P. brassicae* DNA in each sample was estimated using the standard curve. Results were expressed as amount (ρg) of *P. brassicae* DNA in 50 ηg total DNA from each plant sample.

Assessment of P. brassicae *biomass in leaf samples with trypan blue staining*

Leaf discs were decolourised by adding a solution of ethanol and chloroform (3:1, v/v) and stained with 0.025% trypan blue solution for a period of 4 h. Stained leaf discs were each mounted on a glass slide containing a drop of 70% glycerol and covered with a cover slip. The leaf discs were observed under a microscope at ×400 magnification and images were taken for analysis.

Winter oilseed rape field experiments

In the 2015/16 cropping season, a field experiment was established at Rothwell, Lincolnshire including the DH lines used in this study and two commercial oilseed rape cultivars labelled cv1 (susceptible to *P. brassicae*) and cv3 (resistant against *P. brassicae*). Leaves were sampled on 19 November 2015, 19 February 2016, and 4 April 2016 by randomly selecting 6–10 plants from each line and from control cultivars. Each leaf sample was put in a labelled 15 mL Falcon tube, freeze-dried and stored at -20°C for further analysis.

Freeze dried leaf samples of the DH lines studied in the CE experiment and the two control cultivars were selected (Table 1) and leaf samples were ground separately with mortar and pestle until they became a fine powder. A sub-sample of 20 mg was used for DNA extraction. DNA extraction and the quantification of *P. brassicae* in these samples were according to the methods described.

Results

P. brassicae *DNA in leaf samples at different times*

According to the qPCR results for the average quantity of *P. brassicae* DNA in leaf samples collected from the controlled environment experiments at selected times, resistant lines had considerably less *P. brassicae* DNA than susceptible lines and the two susceptible cultivars. There was a considerable increase in the amount of *P. brassicae* DNA in susceptible lines and in the commercial cultivars between 0 and 24 dpi. There was a considerable difference in the amount of pathogen DNA between resistant and susceptible lines at 24 dpi (Table 2).

Table 1. *Number of leaf samples of different lines/cultivars taken from the winter oilseed rape experiment for the quantification of* P. brassicae *by qPCR. The field experiment was established at Rothwell, Lincolnshire in 2015/16 cropping season and leaf samples were collected at three different times*

	Number of samples									
Sample date	R1[a]	R2[a]	R3[a]	R4[a]	S1[b]	S2[b]	S3[b]	S4[b]	cv1[c]	cv3[c]
19 Nov 2015	10	*	*	6	8	6	6	10	6	6
19 Feb 2016	8	4	6	6	7	8	8	8	*	6
4 Apr 2016	6	4	4	8	8	7	6	6	10	8

* No samples were taken; [a] - Lines resistant against *P. brassicae;* [b] - Lines susceptible to *P. brassicae* [c] - Commercial oilseed rape cultivars.

Table 2. *Amount of* P. brassicae *DNA (mean ± SEM) (ρg) in 50 ηg of total DNA extracted from different lines/cultivars from 0–24 days post inoculation. Plants were point inoculated in controlled environment conditions with a* P. brassicae *conidial suspension and leaf samples were taken at five different times (days post inoculation)*

	P. brassicae DNA (ρg)									
Sample date (dpi)	R1[a]	R2[a]	R3[a]	R4[a]	S1[b]	S2[b]	S3[b]	S4[b]	cv1[c]	cv2[c]
Day 0	1.4 ±0.1	*	0.8 ±0.4	0.6 ±0.4	0.3 ±0.1	*	*	1.2 ±3.9	0.2 ±0.1	0.4 ±0.2
Day 3	21.4 ±2.7	0.5 ±0.1	2.5 ±1.3	2.5 ±0.7	3.5 ±0.7	0.6 ±0.1	*	26.1 ±2.7	30.4 ±3.7	4.6 ±0.5
Day 7	34.4 ±19.7	*	15.7 ±3.0	5.5 ±2.9	84.0 ±22.7	1.5 ±0.7	*	60.3 ±18.4	26.3 ±6.0	51.6 ±0.7
Day 13	20.7 ±3.4	*	19.4 ±2.1	14.2 ±3.1	44.8 ±7.0	43.5 ±11.1	*	42.9 ±4.6	176.4 ±41.1	173 ±20.6
Day 24	32.6 ±19.3	29.3 ±9.4	14.7 ±2.1	17.4 ±1.4	60.7 ±5.7	75.2 ±18.7	121.7 ±24.6	57.3 ±11.6	105.4 ±24.6	207.4 ±57.9

* No data available for these sampling times; [a] - Lines resistant against *P. brassicae*
[b] - Lines susceptible to *P. brassicae;* [c] - Commercial oilseed rape cultivars.

P. brassicae *biomass in leaf samples assessed with trypan blue staining*

The microscopic images indicated that there was more *P. brassicae* colonisation in susceptible lines than in resistant lines. According to the observations made at different times, spore germination and penetration was observed by 3 dpi in most of the lines/cultivars and the *P. brasscae* colonisation had started by 7 dpi. There were differences between different lines/cultivars in the number of successful fungal penetrations as well as in the endophytic growth of *P. brassicae*. The resistant lines had little fungal penetration and colonisation. In contrast, the susceptible lines had substantial colonisation by 7 dpi and this increased progressively until 24 dpi. The two commercial cultivars, cv1 and cv2, were observed to have the most pathogen penetration and colonisation.

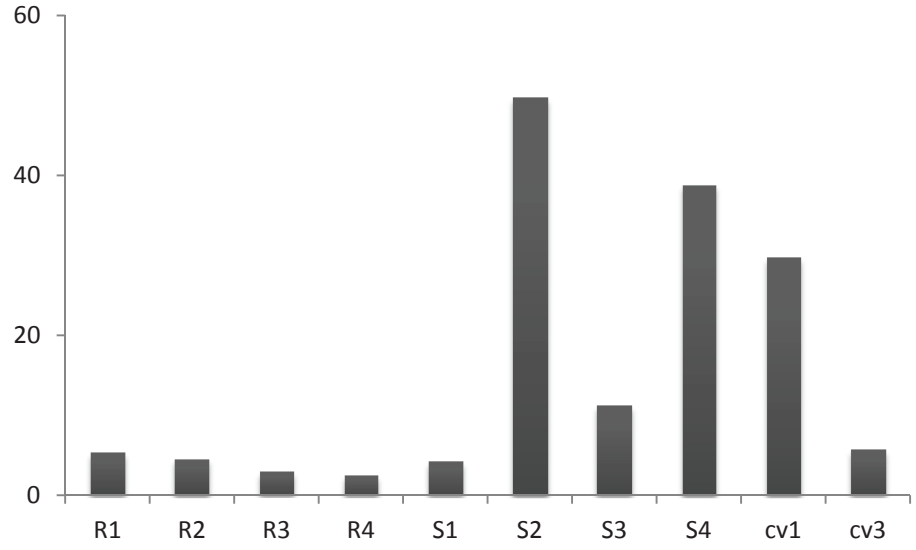

Fig. 1. The amount of *P. brassicae* DNA in the leaf samples of different DH lines and cultivars collected from a winter oilseed rape field experiment in April 2016. (R-resistant lines, S-susceptible lines, cv1-susceptible cultivar and cv3- cultivar resistant against *P. brassicae*).

Winter oilseed rape field experiments

The qPCR results for the amount of *P. brassicae* DNA from the winter oilseed rape experiment samples collected later in the cropping season (4 April 2016) indicated a clear difference between the resistant and susceptible DH lines, cv1 (susceptible cultivar) and cv3 (resistant cultivar). There was more pathogen DNA in susceptible lines except for the line S1 than in resistant lines. The susceptible cultivar cv1 had a considerably greater amount of pathogen DNA compared to the resistant cultivar cv3 (Fig. 1). There was no clear difference between the resistant and susceptible lines in the amount of pathogen DNA in the leaf samples collected at the first two sampling times.

Discussion

The qPCR results for the average quantity of *P. brassicae* DNA in different lines at different sampling times suggested that *P. brassicae* grew less in resistant lines than in susceptible lines. The results were confirmed by the analysis of colonisation by *P. brassicae* using fungal staining of leaf samples at different times, where there was less pathogen biomass in the resistant lines than the susceptible lines. In resistant lines, the amount of pathogen DNA showed little increase from day 0 to day 24. In contrast, there was a considerable increase in pathogen DNA in susceptible lines from 0 to 24 dpi. The leaf samples collected from the winter oilseed rape field experiment in April 2016 also indicated the presence of less *P. brassicae* DNA in resistant lines than in susceptible lines. It was also observed that there were differences between different lines in number of successful infections. However, more research is needed to confirm this observation. It will also be useful to further study these DH lines to identify the effect on other stages of *P. brassicae* life cycle. There are previous studies on resistance against *P. brassicae* (Bradburne *et al*., 1999; Boys *et al*., 2012). Boys *et al*. (2012) reported a resistant phenotype that prevents asexual sporulation of *P. brassicae*. However, the pathogen was able to continue its growth and undergo sexual sporulation after leaf senescence.

In a previous study, different amounts of *P. brassicae* sporulation were observed in different lines from this DH population (K Downes, unpublished data). However, the resistant lines selected for the work described here produced no asexual sporulation. Combination of both visual and molecular assessment methods (such as qPCR) provide insights into the endophytic growth phase of *P. brassicae* and help to identify points at which the host resistance may operate in relation to the pathogen life cycle. Considering the fungal staining and the qPCR data from both controlled environment experiment and the field experiments, it can be suggested that asexual sporulation of *P. brassicae* is interfered with or prevented in the resistant lines by inhibiting the pathogen growth. This type of resistance may also contribute to reduce the amount of pathogen inoculum available. The resistant lines from this population can serve as pre-breeding material to provide a new source of resistance against the light leaf spot pathogen *P. brassicae.* Identification of markers linked to resistance loci can be used to improve the efficiency of breeding programmes.

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